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The Characterization of an Open Reading Frame Involved in Clavulanic Acid Biosynthesis in *Streptomyces clavuligerus*

by

Kenneth James Elder

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Microbiology and Biotechnology

Department of Biological Sciences

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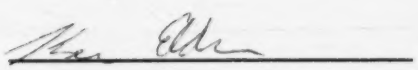

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled The characterization of an open reading frame involved in clavulanic acid biosynthesis in *Streptomyces clavuligerus* submitted by Kenneth James Elder in partial fulfillment of the requirements for the degree of Master of Science in Microbiology and Biotechnology.

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Abstract

A *Streptomyces clavuligerus* gene (designated *orf-2*) located immediately downstream of the cephamycin gene cluster and upstream of the clavulanic acid gene cluster was investigated to determine if it is involved in cephamycin or clavulanic acid biosynthesis. Nucleotide sequence analysis and protein database searching of *orf-2* identified a significant similarity to the large subunit of acetohydroxy acid synthase (AHAS) enzymes which suggested that *orf-2* may be a secondary AHAS enzyme providing valine for the synthesis of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) in cephamycin biosynthesis. A gene replacement mutant disrupted in *orf-2* was constructed to determine the function of *orf-2*. AHAS assays of the *orf-2* mutant determined that AHAS activity was unaltered. Cephamycin levels were also unaltered by the mutation of *orf-2*. Analysis of the *orf-2* mutant for clavulanic acid biosynthesis revealed that clavulanic acid production had been interrupted when grown in both starch-asparagine medium and soy medium. Therefore, *orf-2* appears to be essential for clavulanic acid biosynthesis.

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LIST OF ABBREVIATIONS:

α-aaa	L-α-aminoadipic acid
α-KG	α-Ketoglutarate
AAT	Acyl transferase complex
ACV	δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine
ACVS	ACV synthetase
ACVSR	ACVS related protein
AHAS	Acetohydroxy acid synthase
apra	Apramycin resistance gene
CAD	Clavaldehyde dehydrogenase
CS	Clavaminic acid synthase
DAC	Deacetylcephalosporin C
DACS	Deacetylcephalosporin C synthase
DAOC	Deacetoxycephalosporin C
DAOCS	Deacetoxycephalosporin C synthase
HPLC	High-pressure liquid chromatography
IPN	Isopenicillin N
IPNE	Isopenicillin N epimerase
LAT	L-lysine-ε-aminotransferase
O2Ap	Apramycin disrupted *orf-2*
OCDAC	O-cabamoyl-deacetylcephalosporin C
ORF	Open reading frame
PAH	Proclavaminic acid amidino hydrolase
pbp	Penicillin binding protein
PNS	Isopenicillin N synthase
SA	Starch-asparagine
TSB	Trypticase soy broth
TSBM	TSB + 1% maltose
TSBS	TSB + 1% starch
tsr	Thiostrepton
v/v	Volume/volume
w/v	Weight/volume

I. INTRODUCTION:

Since man first discovered penicillin, extensive research has gone into the discovery of new antibiotics and the organisms which produce them. This led to the discovery of thousands of antibiotics produced by many different species of fungi and bacteria. Although the ability to produce antibiotics is widespread in nature, the vast majority of organisms which produce antibiotics have soil as their habitat, are most efficient in recycling decaying organic materials, and have some sort of differentiation in their life cycle (Lancini and Lorenzetti, 1993). This list includes, starting from the most prolific: the actinomycetes, where the genus *Streptomyces* accounts for three quarters of the producers; the eubacteria, in which the genera *Bacillus* sp. and *Pseudomonas* sp. account for half of the producers; and the lower fungi, where *Penicillium* sp. and *Aspergillus* sp. account for about one third of the producers (Table 1). The *Streptomyces* account for roughly 4500 different antibiotics, many of which are used in medical, agricultural and veterinary practice, while others are used as valuable tools in biochemistry (Hopwood and Chater, 1989). The widespread use of *Streptomyces* antibiotics provides a significant scientific and commercial interest in the further understanding of *Streptomyces* antibiotic biochemistry and genetics.

The actinomycetes are a large group of filamentous bacteria, usually Gram-positive, which form branching filaments called mycelia. Superficially, their morphology resembles that of the filamentous fungi; however, the filaments of actinomycetes consist of procaryotic cells with diameters much smaller than those of molds. Some actinomycetes further resemble molds by using externally carried asexual spores for reproduction. As with fungi, actinomycetes are very common inhabitants of soil, where mycelia have advantages. The organism can bridge water-free gaps between soil particles to move to new nutritional sites. This morphology also gives the organism a much higher surface-area-to-volume ratio and improves its nutritional efficiency in the highly competitive soil environment. The DNA base compositions of all members of the actinomycetes fall within

Table 1: Representative Sources of Antibiotics and their Producers

TYPE	MICROGANISM	ANTIBIOTIC
Actinomycetes		
	Streptomyces parvullus	Actinomycin D
	Streptomyces nodosus	Amphotericin B
	Streptomyces venezuelae	Chloramphenicol
	Saccharopolyspora erythrae	Erythromycin
	Streptomyces fradiae	Neomycin
	Streptomyces griseus	Streptomycin
	Streptomyces clavuligerus	Cephamycin C
	Micromonospora purpureae	Gentamicin
Eubacteria		
	Bacillus subtilis	Bacitracin
	Bacillus polymyxa	Polymyxin
	Bacillus brevis	Tyrothricin
Fungi		
	Cephalosporium sp.	Cephalosporin
	Penicillium griseofulvum	Griseofulvin
	Penicillium chrysogenum	Penicillin
	Penicillium notatum	Penicillin

a relatively narrow range of 63 to 78 percent G + C.

Streptomyces are aerobic Gram-positive filamentous soil organisms belonging to the order Actinomycetales. They are characterized as having a G+ C content of approximately 73 percent. *Streptomyces* filaments are usually 0.5 to 1.0 µm in diameter and of indefinite length, and often lack cross walls in the vegetative stage. *Streptomyces* have a complex life cycle in which mats of branched filaments grow firstly as substrate mycelia which penetrate the soil extracting nutrients. For a large part of their nutrition, they utilize insoluble organic debris by the production of a variety of extracellular hydrolytic enzymes such as cellulases, hemicellulases, amylases, proteases, and nucleases (Hopwood and Chater, 1989). The nutrients thus derived are then used to grow aerial mycelia, which after some physiological switch (e.g. nutrient limitation), are segmented into long chains of spores in a overall process called differentiation. After the switch to differentiation, substrate mycelia begin to lyse, releasing their cellular contents and allowing them to be scavenged by the aerial mycelium for continued growth and formation of spores. Also released about this time are the products of secondary metabolism, such as antibiotics, which may serve as a protection mechanism against other bacteria to prevent them from exploiting the lysing substrate mycelia.

During the early years after the discovery of antibiotics, research was focused on the widespread screening for novel antibiotics and antibiotic producers, which accounts for the vast numbers of antibiotics known to us today, as well as the hundreds of species responsible for their production. Research then shifted to increasing antibiotic production by random mutagenesis, selection of strains with increased production yields, and by empirical modification of fermentation medium composition and growth conditions (A.K. Petrich, 1993). From the mutant strains which produced increased levels of antibiotics, researchers generally found an increased level of the antibiotic biosynthetic enzymes which made the isolation and purification and subsequent biochemical characterization of biosynthetic enzymes for antibiotic production much easier. From the information gathered

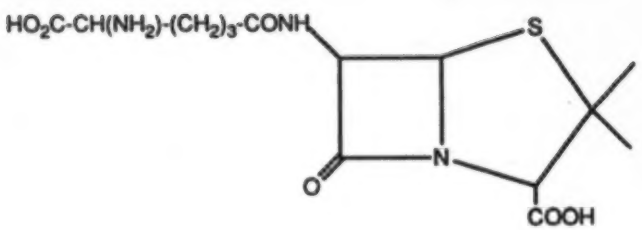
from the purified proteins, the cloning of the corresponding genes was made possible. Current research in *Streptomyces* antibiotic production now seems to be focused on the genetic characterization of antibiotic biosynthetic pathways with an emphasis on known and commercially important antibiotics.

Since the discovery of penicillin nearly seventy years ago, β-lactam antibiotics have remained a very active subject of research both at the industrial and academic level. Their role clinically has remained prominent, in that they still account for greater than 50% of the antimicrobial agents prescribed, making them both medically and economically important (A.K. Petrich, 1993). One model organism widely used for β-lactam research is *Streptomyces clavuligerus.*

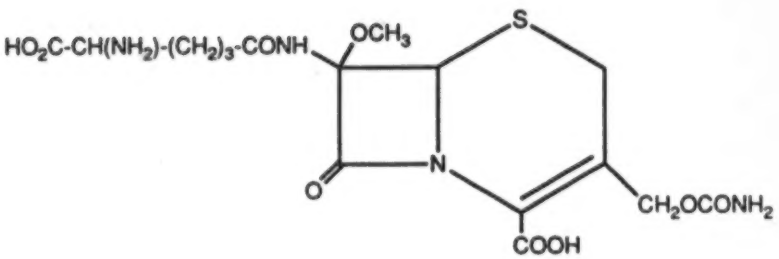
Streptomyces clavuligerus was originally isolated from a South American soil sample (C.E. Higgens and R.E. Kastner, 1971) as a result of the massive screening process used by the major pharmaceutical companies to find new antibiotics. It was selected because it produced a new cephalosporin antibiotic called cephamycin C (cephamycins are an unusual form of cephalosporins that contains a methoxy group attached to the carbon atom at position 7); it also produces penicillin N and was later found to produce the potent β-lactamase inhibitor clavulanic acid (Figure 1). Although the production of the two classical β-lactam antibiotics, penicillin and cephamycin, in *S. clavuligerus* is not commercially important, many have used *S. clavuligerus* as a model for biochemical and genetic analysis of the penicillin and cephamycin pathway. *S. clavuligerus* is now best known as the organism used industrially for the production of clavulanic acid, a potent β-lactamase inhibitor used clinically in combination with β-lactam antibiotics to combat infections caused by β-lactamase-producing organisms.

The branched biosynthetic pathway leading to penicillins, cephalosporins, and cephamycins has now been well characterized; many pathway enzymes have been purified, and corresponding genes have been cloned and sequenced (Jensen and Demain, 1994). As

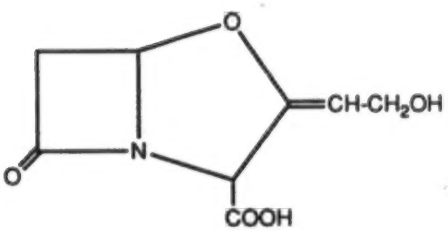
Figure 1: β-lactam antibiotics produced by *Streptomyces clavuligerus*. Penicillin and cephamycin (7-methoxy cephalosporin) are classical β-lactam antibiotics, while clavulanic acid, an oxypenam, is a potent β-lactamase inhibitor.



PENICILLIN N



CEPHAMYCIN C

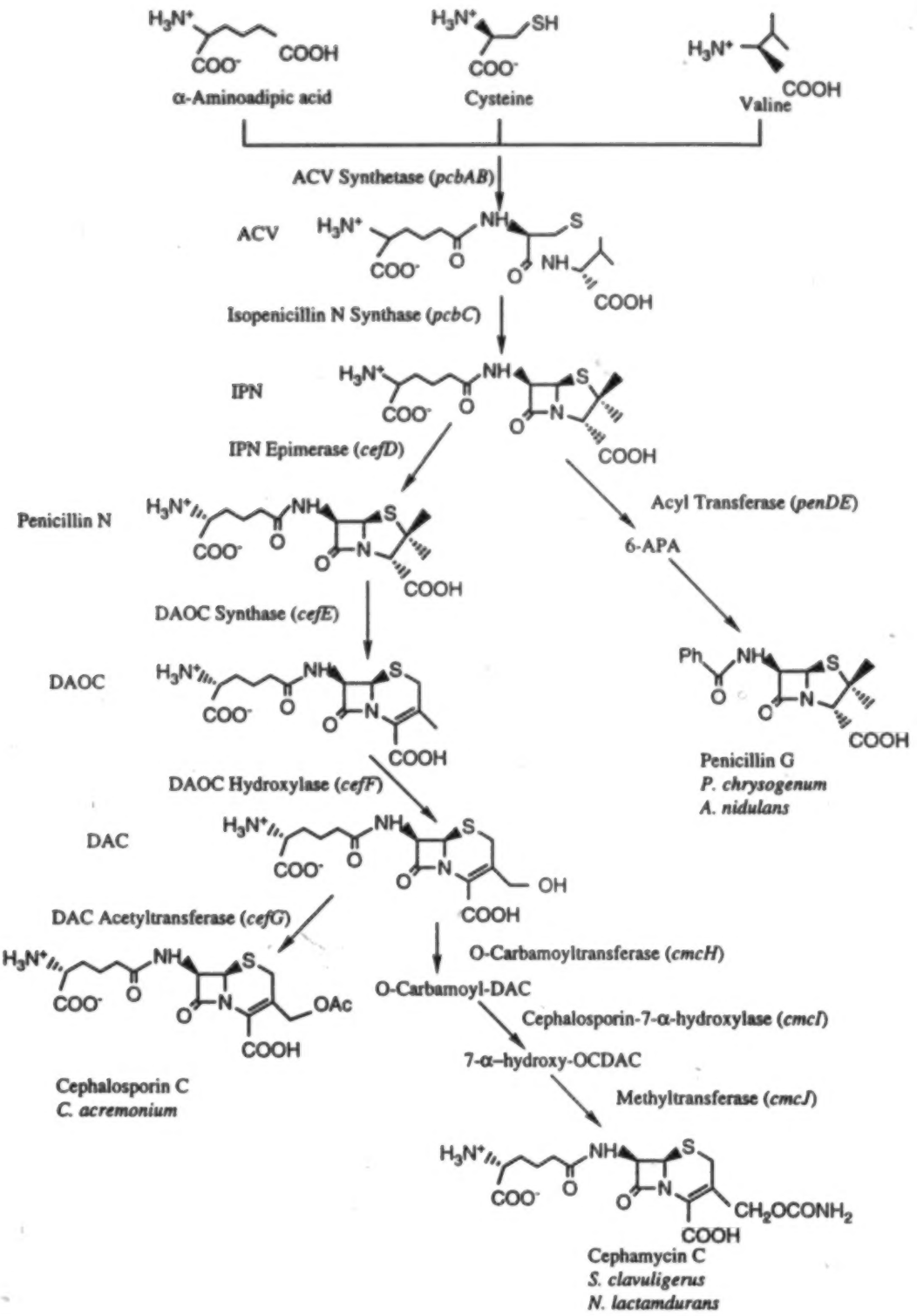


CLAVULANIC ACID

seen in Figure 2, the central pathway common to all three begins with three constituent amino acids, L-α-aminoadipic acid (α-aaa), L-cysteine and L-valine. Cysteine and valine are primary amino acids while α-aaa has different origins in fungi and prokaryotes. In fungi, α-aaa is produced as an intermediate in the biosynthetic pathway to lysine. In actinomycetes on the other hand, there is no primary metabolic pathway to α-aaa, since actinomycetes do not use the α-aaa pathway to lysine. Instead they produce lysine by the pathway via diaminopimelate, as expected for a Gram-positive bacterium. Therefore, in cephamycin-producing *Streptomyces*, a distinctive second pathway for the production of α-aaa must be present. This pathway is driven by L-lysine-ε-aminotransferase (LAT), which catalyzes the removal of the ε-amino group of lysine to form the intermediate 1-piperidine-6-carboxylate. Then 1-piperidine-6-carboxylate undergoes a dehydrogenation step to α-aaa. The gene that encodes LAT has been found linked to the cephamycin gene cluster (Tobin et al., 1991a; Madduri et al., 1991), which supports the concept that LAT catalyzes the reaction specific to the cephamycin biosynthetic pathway.

The condensation of the three amino acids, α-aaa, cysteine and valine, and isomeration of valine to form δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) is the next step. This step is catalyzed by the non-ribosomal peptide synthetase ACV synthetase (ACVS). Isopenicillin N synthase (IPNS) then catalyzes the oxidative cyclization of the linear ACV tripeptide to form the first β-lactam ring-containing intermediate, isopenicillin N (IPN). The next step in the pathway after the formation of IPN is the first branch point in the pathway. Fungal species which only produce penicillin remove the α-aaa side chain of IPN by an acyl transferase complex (AAT) and replace it with non-polar side chains such as phenylacetyl and phenoxyacetyl to produce penicillin G or penicillin V respectively. Fungal and bacterial species which produce cephalosporins and cephamycins instead convert the L-α-aminoadipyl side chain of IPN to the D-form by the action of Isopenicillin N epimerase (IPNE), to produce penicillin N. Penicillin N then undergoes an oxidative

Figure 2: Pathway to cephamycin C biosynthesis in *Streptomyces clavuligerus*. The enzymes and the corresponding genes are indicated at each step.


α-Aminoadipic acid
Cysteine
Valine
ACV Synthetase (pcbAB)
ACV
Isopenicillin N Synthase (pcbC)
IPN
IPN Epimerase (cefD)
Acyl Transferase (penDE)
Penicillin N
6-APA
DAOC Synthase (cefE)
DAOC
Penicillin G
P. chrysogenum
A. nidulans
DAOC Hydroxylase (cefF)
DAC
DAC Acetyltransferase (cefG)
O-Carbamoyltransferase (cmcH)
O-Carbamoyl-DAC
Cephalosporin-7-α-hydroxylase (cmcI)
7-α–hydroxy-OCDAC
Methyltransferase (cmcJ)
Cephalosporin C
C. acremonium
Cephamycin C
S. clavuligerus
N. lactamdurans

ring-expansion of the 5-membered thiazolidine (sulfur containing) ring to a six membered dihydrothiazine ring which is catalyzed by deacetoxycephalosporin C synthase (DAOCS) to form deacetoxycephalosporin C (DAOC). Deacetylcephalosporin C synthase (DACS) then catalyzes the hydroxylation of the C-3 methyl group on the dihydrothiazine ring of DAOC to give deacetylcephalosporin C (DAC).

Once again the pathway diverges depending on the antibiotic-producing species. Those species which produce cephalosporin C, such as *C. acremonium* and *A. nidulans*, complete the pathway from DAC to cephalosporin C in a single step by the action of deacetylcephalosporin C acetyltransferase, which adds an acetyl group to the hydroxyl group on the sulfur-containing ring of DAC. Three reactions remain for the prokaryotes (e.g. *S. clavuligerus* and *Nocardia lactamdurans*) which make cephamycin C. The first of these is a carbamoylation of DAC to O-carbamoyl-deacetylcephalosporin C (OCDAC) by O-carbamoyltransferase, followed by the hydroxylation of OCDAC to form 7-α-hydroxy-OCDAC by the enzyme 7-α-hydroxylase. The final reaction to cephamycin C is the transfer of a methyl group to the C-7 hydroxyl group of 7-α-hydroxy-OCDAC.

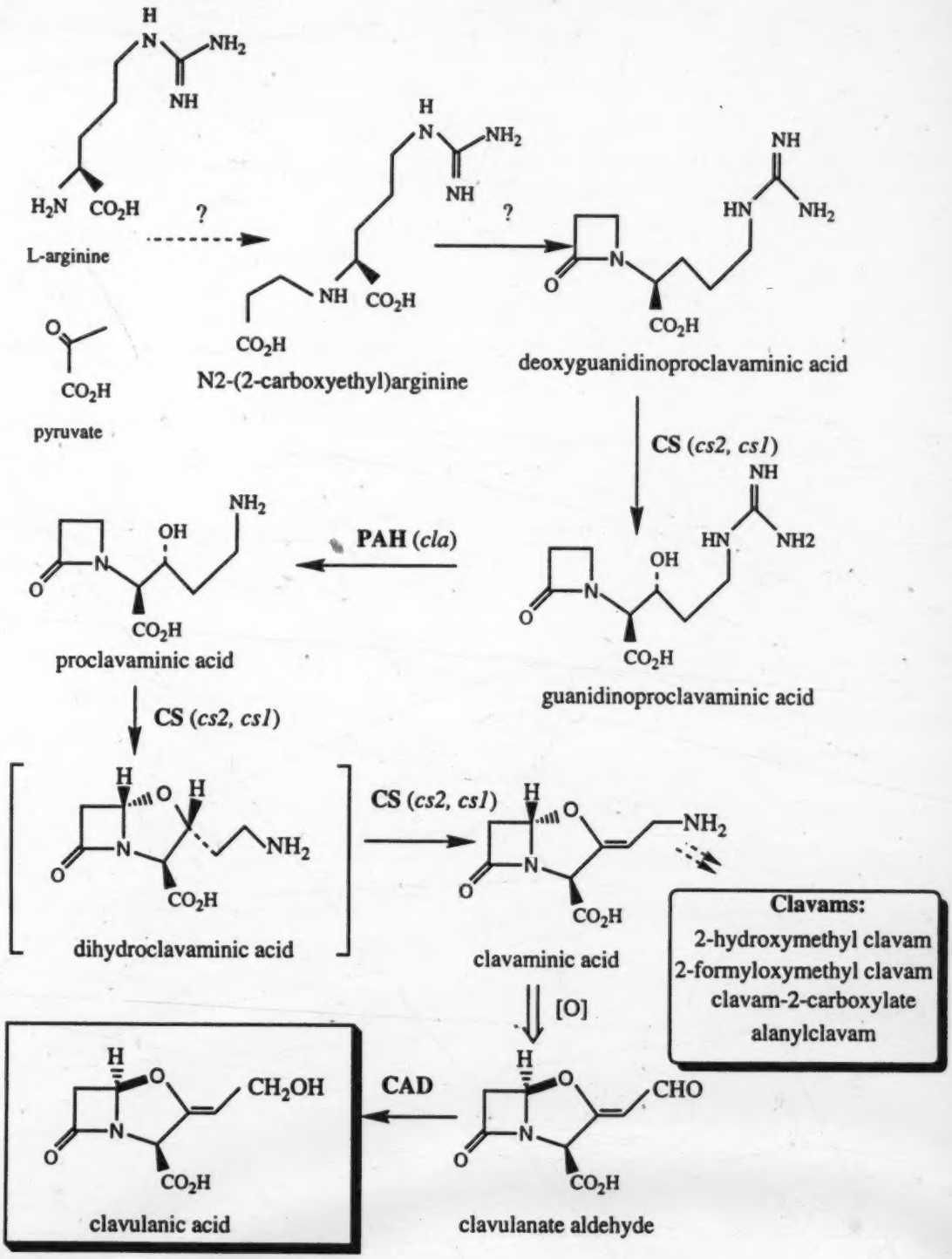
As related above, the biosynthetic pathway of penicillin and cephamycin has been intensively investigated and the central parts of the pathway are now well understood. Also, for the most part, the enzymes have been purified and their genes sequenced and cloned (Aharonowitz et al., 1992; Jensen and Demain, 1994), and in typical fashion for antibiotic biosynthetic genes (Martín, 1992; Seno and Baltz, 1989) they have been found clustered together. Figure 3 shows the approximate location and orientation of the known cephamycin genes from *S. clavuligerus*.

While the biosynthetic pathway for penicillin and cephamycin is known and most of the biosynthetic genes have been cloned and sequenced for some time now, the overall picture for clavulanic acid biosynthesis and genetics is just now being elucidated, with many pieces of the puzzle still missing (Figure 4). Clavulanic acid biosynthesis was originally hypothesized to be interrelated with penicillin and cephamycin biosynthesis due

Figure 3: Linear map of the known cephamycin biosynthetic gene cluster in *S. clavuligerus*. Open boxes represent the approximate sizes of the coding regions for the genes. Arrows indicate the direction of transcription.

cmcT
pcd
cefE
cefD
cmcI
cmcJ
cefF
cmcH
lat
pcbAB
pcbC

Figure 4: Pathway to clavulanic acid biosynthesis in *Streptomyces clavuligerus*. Known steps in the pathway are indicated by solid arrows with corresponding enzymes indicated if determined.


L-arginine
?
N2-(2-carboxyethyl)arginine
?
deoxyguanidinoproclavaminic acid
pyruvate
CS (cs2, cs1)
guanidinoproclavaminic acid
PAH (cla)
proclavaminic acid
CS (cs2, cs1)
dihydroclavaminic acid
CS (cs2, cs1)
clavaminic acid
Clavams:
2-hydroxymethyl clavam
2-formyloxymethyl clavam
clavam-2-carboxylate
alanylclavam
[O]
CAD
clavulanic acid
clavulanate aldehyde

to the conserved β-lactam structure, but it has been shown that they are totally separate pathways (Romero et al., 1984). Clavulanic acid is composed basicly of two building blocks, a five-carbon and three-carbon primary metabolite. The feeding of radioactive labeled ornithine and arginine to auxotrophic mutants of *S. clavuligerus* has shown that ornithine has to be converted to arginine prior to incorporation as the five-carbon precursor into clavulanic acid (Valentine, et al., 1993). Therefore arginine is the more direct precursor to clavulanic acid. Using radiolabeled precursors, it was shown that pyruvate is the most likely three-carbon precursor of clavulanic acid in studies by Thirkettle et al. (1997). Elson, et al. (1993c) using labeled precursors and a mutant blocked in clavulanic acid biosynthesis identified the first arginine derivative, N2-(2-carboxyethyl)arginine, in the pathway to clavulanic acid, as well as the next two intermediates in the pathway; 5-guanidino-(2-oxoazetidin-1yl)pentanoic acid (deoxyguanidinoproclavaminic acid) and 3-hydroxy-5-guanidino-2-(2-oxoazetidin-1-yl)pentanoic acid (guanidinoproclavaminic acid) respectively. Deoxyguanidinoproclavaminic acid, the first cyclic intermediate in the pathway, is hydroxylated by the α-ketoglutarate (α-KG) dependent oxygenase clavaminic acid synthase (CS)(discussed later) to guanidinoproclavaminic acid (Elson, et al., 1993c; Baldwin, et al., 1993).

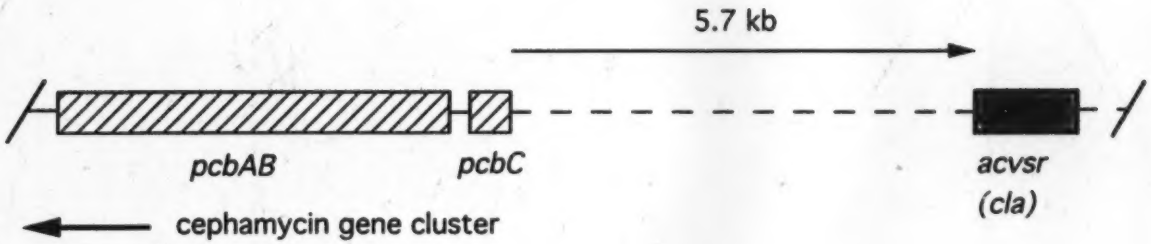
The mutant responsible for the discovery of the previous three intermediates (Elson, et al., 1993b,c), was found to be deficient in the enzyme responsible for the next step in the pathway, which hydrolyzes guanidinoproclavaminic acid to proclavaminic acid and urea (Elson, et al., 1987). From what was at the time unpublished genetic evidence which supported this hypothesis, Elson, et al. (1993c) proceeded to isolate an enzyme with a molecular weight of 33 000 which converts guanidinoproclavaminic acid to proclavaminic acid. The N-terminal amino acid sequence of the enzyme, which was named proclavaminic acid amidino hydrolase (PAH), correlated with the open reading frame of the amidino hydrolase-related gene located in the clavulanic acid genetic cluster.

Roughly parallel to the work done by Elson's group on identifying early intermediates in the clavulanic acid pathway and the identification of PAH, the group of Jensen et al. found the gene which corresponds to PAH. During purification studies on ACVS, the non-ribosomal peptide synthetase responsible for an early step in the penicillin/cephamycin biosynthetic pathway, a second protein co-purified with ACVS (Jensen et al., 1990). It was thought that since the second protein co-purified with ACVS, the two proteins were somehow associated, thus the new protein was named ACVS related (ACVSR) protein. Since the genes involved in penicillin and cephamycin biosynthesis are clustered, it was thought that if ACVSR was also involved in penicillin and cephamycin biosynthesis, the gene which encodes ACVSR (*acvsr*) would be expected to be closely linked to the penicillin/cephamycin cluster. Aidoo, et al. (1993) then determined where *acvsr* mapped. An oligonucleotide probe was made based on the N-terminal amino acid sequence of ACVSR and then used to screen a library of *S. clavuligerus* DNA. The *acvsr* gene in *S. clavuligerus* was found to be located about 5.7 kb downstream from the end of the known penicillin/cephamycin biosynthetic cluster (Figure 5).

The close linkage of *avcsr* to the penicillin/cephamycin cluster promoted further study. The *acvsr* gene was sequenced and the corresponding amino acid sequence was deduced (Jensen et al., 1993; Aidoo et al, 1993). When this deduced amino acid sequence was compared to protein sequence databases, it showed the highest similarity to enzymes involved in arginine metabolism, notably agmatine ureahydrolase and arginases. This seemed more consistent with clavulanic acid production than penicillin/cephamycin biosynthesis since at the time, arginine and ornithine were considered immediate precursors to clavulanic acid (Romero et al., 1986).

When *acvsr* was used to probe genomic DNA of related species, cross-reactivity was found with other species which produced clavam metabolites, while no cross-reactivity was found with species which produced only penicillin and cephamycin. Involvement of ACVSR in clavulanic acid production was finally proven by gene disruption experiments in

Figure 5: Location of the *acvsr* (*cla*)gene relative to the cephamycin biosynthetic gene cluster.


5.7 kb
pcbAB
pcbC
acvsr
(cla)
cephamycin gene cluster

which the wild-type copy of the gene was replaced by a copy that has had its open reading frame (ORF) disrupted by insertion of an antibiotic resistance gene. Disruption of the *acvsr* gene in this way blocked clavulanic acid production in starch-asparagine medium, strongly suggesting an essential role for ACVSR in clavulanic acid production and thus providing the first direct evidence of a gene involved in clavulanic acid biosynthesis. In view of its relevance to clavulanic acid biosynthesis, *acvsr* was renamed *cla*.

PAH, which corresponds to the *cla* gene product, was further characterized by Wu, et al. (1995). They over-expressed the putative open reading frame to produce a catalytically active protein and determined that: PAH has a requirement for Mn^{2+} ions, alkaline pH optimum, and apparent Km in the millimolar range, which is similar to other enzymes of the arginase family (Ouzounis and Kyrpides, 1994).

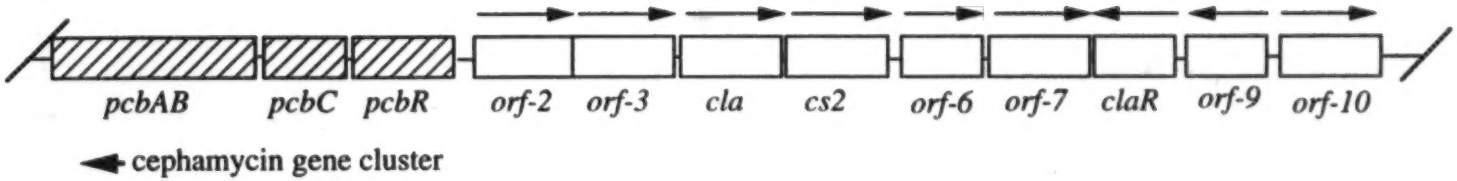
In the next step in the biosynthetic pathway, proclavaminic acid serves as a substrate for clavaminic acid synthase (CS) which oxidatively catalyzes the formation of the oxazolidine ring resulting in the formation of clavaminic acid. These two intermediates and the enzyme (CS) that mediates the reaction from one to the other were the first steps in the clavulanic acid biosynthetic pathway to be elucidated (Elson, et al., 1987). Later it was shown that CS mediates a stepwise pair of oxidative reactions from proclavaminic acid to clavaminic acid, with dihydroclavaminic acid being the intermediate (Baldwin, et al., 1990; Salowe, et al., 1991). During more intensive purification studies on CS there were indications of two forms of the enzyme being present in *S. clavuligerus* (Salowe, et al., 1991). The gene for each has since been cloned and sequenced, and found to be significantly homologous (87% identity) (Marsh, et al., 1992), with the two corresponding genes, *cs1* and *cs2*, having open reading frames of 975 and 978 nucleotides, respectively, encoding proteins of M of 35 347 and 35 774. These genes were identified by screening a cosmid library of *S. clavuligerus* DNA. From this method of identification, it was concluded that they were separated by >20kbp, since there were no overlapping cosmid

clones containing *cs1* or *cs2*. More recently, Busby, et al. (1995) have over-expressed both *cs1* and *cs2* in *Escherichia coli* allowing preliminary investigation of the active site.

Jensen et al., intrigued by the unexpected finding of the *cla* gene which encodes proclavaminic amidino hydrolase (PAH), examined the region of the chromosome flanking *cla* further (Jensen et al, 1993; Aidoo et al, 1993). The DNA sequence was determined starting from the last known penicillin/cephamycin gene (*pcbC*) and continuing downstream for about 15 kb. It was found that this area contained a total of ten open reading frames (ORFs), with *orf-4* corresponding to *cla* (Figure 6). ORFs 2 to 9 were then cloned into a high copy number shuttle vector and transformed into *Streptomyces lividans* (a species which does not produce clavam metabolites). The introduction of these genes resulted in the production of a β-lactamase inhibitory activity. Based on this result it was concluded that ORFs 2 to 9 must encode all of the structural genes necessary for the production of clavulanic acid (Aidoo et al., 1993).

Comparison of the predicted amino acid coding sequences of the newly determined ORFs to protein sequence databases showed varying degrees of similarity to proteins of known function. ORF 1 shows some similarity to penicillin binding proteins from species which are resistant to β-lactam antibiotics and has since been shown to be a penicillin binding protein involved in self-resistance and thus named *pbpR*. ORF 2 shows a high degree of similarity to acetohydroxy acid synthase (AHAS) enzymes from various sources. ORF 3 and 5 did not show any significant similarity to any known proteins in the original search, although it was later found that *orf-5* was the gene which encodes CS2 (Marsh et al., 1992). ORF 6 showed similarity to ornithine acetyltransferase, which possibly indicates an association with clavulanic acid production since there is an ornithine-like residue in a precursor to clavulanic acid. ORF 7 shows weak similarity to protein XP55 from *S. lividans*, and a lower level of similarity to oligopeptide-binding proteins from various species. ORF 8 showed a weak similarity to several transcriptional activator proteins and has since been determined to be *claR*, a pathway-specific transcriptional

Figure 6: Open reading frames surrounding the *cla* gene. The direction of transcription is indicated by the arrows. Hatched boxes represent open reading frames from the cephamycin gene cluster. Open boxes represent the putative clavulanic acid gene cluster.


pcbAB
pcbC
pcbR
orf-2
orf-3
cla
cs2
orf-6
orf-7
claR
orf-9
orf-10
cephamycin gene cluster

activator which regulates the late steps of clavulanic biosynthesis (Paradkar et al., 1998). ORF 9 shows weak similarity to ribitol-5-phosphate dehydrogenase-type enzymes. ORF 10 shows strong similarity to cytochrome P450 type enzymes, but this gene lies outside the fragment which conferred β-lactamase inhibitory ability in *S. lividans*, so it may not be required for clavulanic acid synthesis (Jensen, et al. 1993).

From the above information generated from the unexpected isolation of the PAH protein and the identification of its corresponding gene (*cla*), great interest was generated regarding the role of surrounding open reading frames. From the results of Marsh et al. (1992), it was evident that ORF 5 corresponded to the gene encoding *CS2*. Paradkar and Jensen (1995) developed a *S. clavuligerus* mutant disrupted in *cs2* by a gene replacement procedure. They determined that the *cs2* mutant did not produce any clavulanic acid while grown in starch-asparagine medium, while the *cs2* mutant did produce clavulanic acid, although at reduced levels compared to wild-type cultures, when grown in soy medium. Transcriptional analysis of *cs1* and *cs2* supported this medium-dependent leaky phenotype. The *cs1* transcript, encoding the CS1 enzyme, was present when the mutant was grown on soy medium, while it was absent in starch-asparagine medium. Under nutritional conditions in which *cs1* expression is blocked, *cs2* becomes essential for clavulanic acid production (Paradkar and Jensen, 1995). As well, they determined that *cs2* is transcribed as both a 1.2-kb monocistronic transcript and as part of a 5.3-kb polycistronic message. The size of the longer transcript suggests that it could accommodate *cs2*, *cla*, and two open reading frames further upstream (*orf-2* and *orf-3*) (Paradkar and Jensen, 1995).

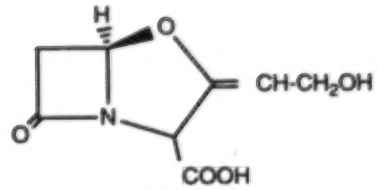
In another confirmation that the region around *cla* and *cs2* encodes genes responsible for clavulanic acid, Hodgson et al. (1995) published the nucleotide sequence of a DNA fragment containing five open reading frames, corresponding to *orf-3*, *cla*, *cs2*, *orf-6*, and *orf-7*, which complemented different clavulanic acid non-producing mutants. The most recent information on the further identification of genes involved in clavulanic acid production is the identification of a pathway-specific transcriptional activator (ClaR)

corresponding to *orf-8*, which regulates the late steps of clavulanic acid biosynthesis in *S. clavuligerus* (Paradkar et al., 1998).

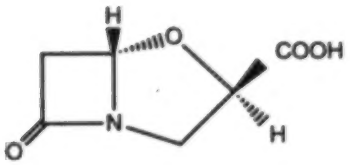
Clavaminic acid represents a branch-point in the biosynthetic pathway to clavulanic acid and the other clavam metabolites (oxygen-containing β-lactam compounds structurally related to clavulanic acid, but differing in bioactivity) produced by *S. clavuligerus* (Egan et al., 1997). These clavams (Figure 7) include 2-hydroxymethyl clavam, 2-formyloxymethyl clavam (2-hydroxyethyl clavam), and clavam-2-carboxylate (Brown et al., 1979), which have antifungal activities, and alanylclavam (Pruess and Kellett, 1983), which has antifungal as well as antibacterial properties (Paradkar and Jensen, 1995). Original indications that the pathways to clavulanic acid and the other clavams were linked came with feeding experiments with radioactively labelled proclavaminic acid, which was equally incorporated at the C-2 position of both clavulanic acid and clavam-2-carboxylate (Iwata-Reuyl and Townsend, 1992). Other evidence which supports this hypothesis is that *Streptomyces antibioticus*, a clavulanic acid non-producer, which produces two clavam metabolites (valclavam and 2-hydroxyethyl clavam), but not clavulanic acid, was shown to incorporate proclavaminic acid into valclavam and 2-hydroxyethyl clavam (Janc et al., 1993). Therefore, it would appear that all clavams and clavulanic acid have a common biosynthetic origin at least up to and including proclavaminic acid.

S. antibioticus has also been shown to produce isozymes of CS and PAH (Baldwin et al., 1994). As well, Janc et al., (1995) purified a CS isozyme from *S. antibioticus* which shows high levels of homology and identical activity to CS1 and CS2 of *S. clavuligerus*. With the presence of CS in *S. antibioticus*, it was reasonable to assume that the CS enzyme has the capability to convert proclavaminic acid to clavaminic acid. Since *S. antibioticus* does not produce clavulanic acid, it seemed plausible that clavaminic acid may also be a intermediate in the pathway to valclavam and 2-hydroxyethyl clavam. Using labeled clavaminic acid, it was shown that clavaminic acid is efficiently incorporated into

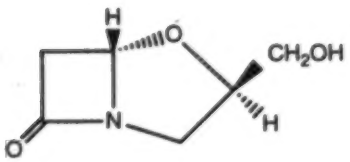
Figure 7: Clavam metabolites produced by *S. clavuligerus*. Clavulanic acid differs from 2-hydroxymethyl clavam, 2-formyloxymethyl clavam, clavam-2-carboxylate, and alanylclavam by the opposite stereochemical configuration at the ring junction and the lack of a C-3 carboxyl group.



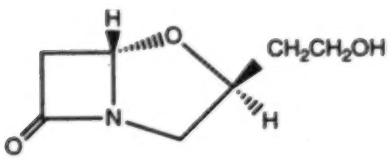
CLAVULANIC ACID



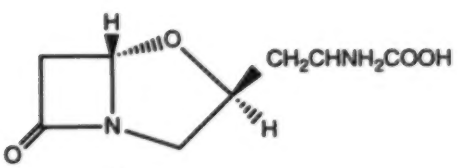
CLAVAM 2-CARBOXYLATE



2-HYDROXYMETHYLCLAVAM



2-FORMYLOXYMETHYLCLAVAM



ALANYLCLAVAM

valclavam and 2-(2-hydroxyethyl) clavam equally as well as proclavaminic acid, indicating that clavaminic acid is the final intermediate shared in the pathway to clavulanic acid and the clavams (Egan et al., 1997).

In the final steps in the clavulanic acid biosynthetic pathway, from clavaminic acid to clavulanic acid, clavaminic acid undergoes a oxidative deamination and enantiomerization (Townsend and Krol, 1988) to the aldehyde, which is then reduced by the NADPH-dependent clavulanate aldehyde dehydrogenase (CAD) to clavulanic acid (Nicholson et al., 1994). These late steps have been recently found to be regulated by a transcriptional regulator encoded by the gene *claR* (Paradkar et al., 1998). A gene replacement mutant disrupted in *claR* was unable to produce clavulanic acid and consequently accumulates clavaminic acid. Transcriptional analysis showed that the *claR* mutant no longer expressed the putative late genes *orf-7*, *orf-9*, and *orf-10*, confirming the regulatory role of *claR*. The putative protein encoded by *orf-7* shows similarity to oligopeptide transport proteins and therefore could be involved in the transport of clavulanic acid (Hodgson et al., 1995), while those encoded by *orf-9* and *orf-10* resemble dehydrogenases and cytochrome P450 hydroxylases, respectively, and could be involved in the conversion of clavaminic acid to clavulanic acid (Paradkar, Aidoo, and Jensen, unpublished).

The focus of the research being presented here is the further investigation of *orf-2* of the putative clavulanic acid gene cluster. When this project was conceived, little was know about the clavulanic biosynthetic pathway and gene cluster. The unexpected purification of PAH (Jensen et al., 1990) and the identification of its gene (*cla*) Aidoo et al., 1993; Jensen et al, 1993; Aidoo et al., 1994) stimulated the investigation of the open reading frames surrounding *cla*. Since *orf-2* is as closely linked to the clavulanic acid biosynthetic cluster as it is to the penicillin/cephamycin biosynthetic cluster, and due to the natural clustering of genes for β-lactam biosynthesis (Ward and Hodgson, 1993), *orf-2* could belong to either pathway with equal likelihood.

The deduced protein sequence encoded by *orf-2* shows a high degree of similarity to the large or α subunit of AHAS enzymes (Table 2). AHAS catalyses the first common step in the branched-chain amino acid pathway which produces valine, isoleucine and leucine. AHAS catalyzes two parallel reactions: the irreversible decarboxylation of pyruvate and its condensation with a second molecule of pyruvate to form acetolactate in the pathway to valine and leucine; and the irreversible decarboxylation of pyruvate and its condensation with α-ketobutyrate to form acetohydroxybutyrate in the pathway to isoleucine. The biosynthetic pathways of amino acids are generally highly regulated; in the case of valine, isoleucine and leucine production, the main regulatory step is at the level of AHAS. In most species AHAS is regulated by feedback inhibition by valine, and in some cases by isoleucine and leucine as well (Riccardi et al., 1989).

There seems to be no existing literature on AHAS activity in *S. clavuligerus*, although there has been extensive studies on AHAS enzymes in many other species. In enteric bacteria there are three isozymes, each composed of a large (α) and small (β) subunit. It has generally been accepted that the large subunits are catalytic, while the small ones are regulatory (Weinstock et al, 1992). The three isozymes are differentiated by their ratio of relative affinities for α-ketobutyrate over pyruvate (R value); for isozyme III, R=20; isozyme II, R=65 to 85; and isozyme I, R=1 to 2. The catalytic activity of isozyme I is dependent on flavin adenine dinucleotide (FAD). Isozymes I and III are inhibited 90% by valine at 1.5 mM and 45% by 3 mM isoleucine, whereas isozyme II is resistant to branched chain amino acid inhibition.

Corynebacterium glutamicum, a more closely related species to *Streptomyces* spp., has only a single AHAS enzyme present with a relative affinity for α-ketobutyrate about three times higher than that of pyruvate. The higher affinity for α-ketobutyrate would seem to favour the formation of α-acetohydroxybutyrate for isoleucine synthesis rather than α-acetolactate for valine synthesis, although this may not be the case since the pool of α-ketobutyrate is controlled by threonine deaminase. This enzyme is inhibited by all three

Table 2: A selection of proteins homologous to the putative amino acid sequence of *orf-2*. The amino acid sequence was compared using a Gapped BLAST search (Blast 2.0) from NCBI. Acetolactate synthase and Acetohydroxy acid synthase (AHAS) describe the same enzyme. The alpha and large subunits of AHAS are synonomous.

Protein Homologous to ORF-2	Organism	% Identity	% Positives
alpha-acetolactate synthase protein	*Bacillus subtilis*	27%	45%
acetolactate synthase	*Synechocystis sp.*	29%	44%
AHAS, large subunit	*Methanococcus jannaschii*	28%	45%
AHAS, large subunit	*Methanococcus aeolicus*	27%	45%
alpha-acetolactate synthase	*Lactococcus lactis*	27%	46%
acetolactate synthase, large subunit	*Methanobacterium thermoautotrophicum*	26%	44%
AHAS, large subunit	*Spirulina platensis*	28%	43%
AHAS, large subunit	*Klebsiella pneumoniae*	25%	43%
AHAS, large subunit	*Corynebacterium glutamicum*	27%	43%
alpha-acetolactate synthase	*Oenococcus oeni*	24%	43%
AHAS isozyme III large subunit	*Escherichia coli*	25%	42%
AHAS, large subunit	*Porphyra purpurea*	25%	42%
acetolactate synthase, large subunit	*Archaeoglobus fulgidus*	25%	41%
AHAS, large subunit	*Streptomyces avermitilis*	26%	41%
pyruvate oxidase	*Streptococcus pneumoniae*	21%	42%
pyruvate dehydrogenase	*Escherichia coli*	21%	36%

branched chain amino acids, but inhibition does not exceed 50% even with valine which is the best effector (Eggeling et al. 1987).

More recently the *ilvBN* gene encoding the large and small subunit of AHAS from *Streptomyces avermitilis* was cloned, sequenced and expressed by De Rossi et al. (1995). The study was motivated around the fact that *S. avermitilis* produces avermectins, antiparasitic compounds, which are derived from common intermediates of the branched chain amino acid pathway. The AHAS of *S. avermitilis* is encoded by two genes (*ilvB* and *ilvN*), as is the case for all other bacterial biosynthetic AHAS enzymes (Umbarger, 1987). The AHAS large and small subunits are most similar to the homologues from *C. glutamicum* with 60% and 44% identity respectively. Notably, the *ilvB,N* and *C* (encoding acetohydroxy acid isomoreductase) genes are found clustered, with *ilvB* and *N* having very little intervening sequence as was found with other species investigated so far. They also noted that AHAS activity was only weakly feedback-inhibited by valine.

The sequence similarity of ORF-2 to AHAS enzymes suggests it could be part of the penicillin/cephamycin pathway. This suggestion seems plausible since AHAS is required for the production of valine, an essential component of penicillin and cephamycin. It has been hypothesized (S.E. Jensen, personal communication) that ORF-2 could be part of a secondary AHAS responsible for the flow of valine into the penicillin/cephamycin biosynthetic pathway; for example, the relative affinity for pyruvate may be higher in a AHAS isozyme needed for increased biosynthesis of valine as opposed to recruitment of α-ketobutyrate and pyruvate into the isoleucine pathway. It was shown by Mendelovitz and Aharonowitz (1982) that valine was not limiting in the production of cephamycin C in a glycerol-asparagine medium, however strains of *S. lipmanii* defective in the control of the isoleucine-valine biosynthetic pathway proved to be superior producers of cephamycins (Godfrey, 1973). Godfrey (1973) then suggested that feedback mechanisms may cause valine to be a rate limiting precursor in the wild-type. In valine auxotrophs, supplementation of valine above the minimum that allowed good growth was necessary

before penicillin was produced, indicating that the amino acid was recruited preferentially into protein synthesis. In studies of species which are high producers of penicillin or cephamycin, the presence of AHAS enzymes at high concentration or with defective or reduced control were common (Vining et al., 1990). Therefore, *S. clavuligerus* may have some mechanism, such as ORF-2 as part of a second AHAS, which circumvents the problem of valine limitation in penicillin/cephamycin production.

As stated above, it is equally plausible that the function of ORF-2 may lie in the clavulanic acid pathway. One piece of circumstantial evidence which suggests that *orf-2* may be involve in clavulanic acid production is that when a plasmid containing a 12-kb *Eco*RI fragment of DNA containing ORFs 2-9 was inserted into *Streptomyces lividans*, it conferred β-lactamase inhibitory ability (Jensen et al, 1993; Aidoo et al., 1993). Another supporting piece of evidence is that of these nine ORFs, the first seven are all orientated in the same direction, and the close spacing of ORFs 2 and 3 and ORFs 4 and 5 suggests that they may be transcriptionally linked. Further work on *orf-5* reaffirmed this hypothesis. Transcriptional analysis of *orf-5* (*cs2*) determined that *cs2* is transcribed as both a 1.2-kb monocistronic transcript and as part of a 5.3-kb polycistronic message. The size of the longer transcript suggests that it could accommodate *cs2*, *cla*, and two open reading frames further upstream (*orf-2* and *orf-3*) (Paradkar and Jensen, 1995). As well, the 5.3-kb message suggests that under some conditions, *orf-2* may be under the same transcriptional control as the `early´ clavulanic acid pathway genes *cla* and *cs2*.

In order to determine the function of *orf-2*, an approach similar to that employed by Jensen et al.(1993) in their determination of the function of the *acvsr* gene was envisioned. In their studies the *acvsr* gene was cloned into a shuttle vector and a marker gene (thiostrepton resistance) was inserted within the coding sequence. The insertion of the marker gene within *acvsr* disrupted the ORF causing a mutation in *acvsr* as well as providing a marker (i.e. thiostrepton resistance) indicating that the ORF has been disrupted. The shuttle vector carrying the disrupted gene was then introduced into *S. clavuligerus.*

Thiostrepton resistant transformants were selected followed by subculture onto medium containing low levels of thiostrepton. Growth on low levels of the antibiotic allowed the isolation of strains in which free plasmid was eliminated from the cells, and the original chromosomal copy of *acvsr* was replaced by a double cross-over recombinational event, with a thiostrepton-interrupted copy (Jensen et al, 1993b). It was then found, by using a bioassay procedure (Jensen et al, 1982), that the mutated strain (*acvsr*) was deficient for clavulanic acid production .

A similar gene disruption method was envisioned for the disruption of *orf-2*. An *S. clavuligerus* strain carrying a mutated copy of *orf-2* could then be assayed for any change in AHAS activity, penicillin/cephamycin production, clavulanic acid production or clavam production. The specific inactivation of *orf-2* was accomplished by the insertion of a non-homologous DNA sequence within the reading frame of a cloned copy of *orf-2*. In this case, the insertion sequence was an apramycin resistance gene which provides a convenient marker to follow the inactivated copy of *orf-2*. The disrupted copy of *orf-2* was then inserted into a segregationally unstable *Streptomyces* plasmid vector and transformed into wild-type *S. clavuligerus*. After selection of primary transformants, the transformants are repatched and allowed to sporulate. This extra round of growth without antibiotic selection allowed homologous recombination and crossing-over of the disrupted *orf-2* plasmid copy with the wild-type *orf-2* to occur, while sporulation cures the transformants of plasmid. Then after serial dilution and plating for isolated colonies, the colonies were replica-plated onto medium containing apramycin and medium containing thiostrepton. When transformants are growing on plates with no thiostrepton there is no selection for the plasmid vector, while apramycin selects for the *apr* gene within *orf-2*. Replica-colonies which have an apramycin resistant colony and no corresponding thiostrepton resistant colony have most likely undergone homologous recombination and crossing-over of the *apr* disrupted *orf-2* plasmid copy with the wild-type *orf-2*. This event was examined for by Southern blot analysis of the appropriately restriction enzyme digested genomic DNA from

the *orf-2* mutant and comparing to the wild-type. If the correct crossing over occurs and the plasmid has been cured from the transformant, a single *orf-2*- specific band should be present that has increased in size by the size of the *apr* insert. As a confirmation of the replacement of *orf-2* with the *apr* disrupted *orf-2*, the same blot could be probed with an *apr* probe. The *apr* probe should hybridize to the same sized band as the *orf-2* probe.

Our assumption was that disruptional inactivation of *orf-2* would cause a deficiency in whatever function *orf-2* is responsible for. We could therefore compare the phenotype of the *orf-2* mutant to wild-type *S. clavuligerus* in order to determine the function of *orf-2*. We know from preliminary evidence that the predicted protein sequence of *orf-2* resembles AHAS enzymes and that *orf-2* is closely linked to both clavulanic acid and cephamycin biosynthetic genes, therefore analysis of the mutant for altered AHAS activity, clavulanic acid production, and cephamycin production is the logical starting point for examining the *orf-2* muntants. If *orf-2* is an AHAS enzyme involved in penicillin and cephamycin production, AHAS activity and possibly cephamycin production would be altered. On the other hand, if *orf-2* is involved in clavulanic acid production, there may be a disruption in the biosynthetic pathway to clavulanic acid which would result in altered clavulanic acid production.

II. MATERIALS AND METHODS

II.1 MATERIALS

Restriction endonucleases were obtained from either Bethesda Research Laboratories, Inc., Burlington, Ont. or Boehringer Mannheim, Laval, Quebec. T4 DNA ligase, T4 polynucleotide kinase from T4-infected *Escherichia coli* B cells, and *E. coli* DNA polymerase I were obtained from Boehringer Mannheim. All enzymes were used according to specifications advised by the manufacturer.

Deoxyribonucleoside triphosphates were purchased from Boehringer Mannheim.

Radioactively labeled [α-^{32}P]dCTP was obtained from ICN Biochemicals, St.-Laurent, Quebec.

The oligonucleotide linker used to insert a *NcoI* restriction site within the *NotI* site of *orf-2* was obtained from the Dept. of Microbiology DNA Synthesis Laboratory, University of Alberta, Edmonton, Alberta, Canada.

Clavam-2-carboxylate, clavaminic acid and clavulanic acid standards were gifts from SmithKline Beecham, Worthing, U.K.

ISP-3, Trypticase Soy Broth (TSB), Yeast extract, Bacto peptone, nutrient broth and malt extract were purchased from Difco Laboratories, Detroit, Michigan, USA.

All other chemicals were reagent grade.

II.2 BACTERIAL STRAINS, PLASMIDS, AND CULTURE CONDITIONS

Streptomyces clavuligerus NRRL 3585 (Northern Regional Research Center, Peoria, Ill.) and *Streptomyces lividans* TK24 (Kieser et al., 1982) were maintained as frozen glycerol stocks of spores. *S. clavuligerus* was grown on sporulation media as described by Jensen et al. (1982b) and spores were prepared as described by Petrich (1993). Spore stocks of *S. lividans* TK24 were prepared as described previously (Hopwood et al., 1985).

The *Streptomyces* plasmid pIJ486, a 6.2 Kb high copy number vector which replicates autonomously in most *Streptomyces* species, was provided by D. A. Hopwood, John Innes Inst., Norwich, U.K.

S. clavuligerus and *S. lividans* were grown for the purposes of plasmid isolation according to Hopwood et al. (1985). For small scale preparations, cells were grown in 10 mL of trypticase soy broth (TSB) + 1% maltose (TSBM) containing either thiostrepton (25 µg/mL, *S. lividans*; 5 µg/mL, *S. clavuligerus*) or apramycin (25 µg/mL for both species) where appropriate. For large scale preparations, a 25 mL TSBM seed culture with the appropriate antibiotic selection was started from spores and grown for 48 hr and subcultured at 2% (v/v) into 100 mL of TSBM and further incubated for 48 hr.

For preparation of cell free extracts for enzyme assays, *S. clavuligerus* spores were inoculated into a 25 mL TSB + 1% starch (TSBS) seed culture and grown for 48hr and subcultured at 2% (v/v) into either 100 mL starch-asparagine (SA) media (Aharonowitz and Demain, 1978) or 100 mL soy media [1.5% (w/v) soy flour, 4.7% (w/v) starch, 0.01% (w/v) KH_2PO_4, 2 mL of filter sterilized $FeSO_4 \cdot 7H_2O$ (0.01g/mL H_2O), pH 6.8]. Cultures were further incubated at 28°C and 250 rpm for the stipulated time periods before harvesting.

For the purpose of generating protoplasts, *S. clavuligerus* spores were used to inoculate 25 mL TSB + 1% maltose followed by incubation at 28°C and 250 rpm. After 48 hr incubation, 1 mL of this seed culture was transferred to a 25 mL TSB/YEME [10 mL TSB/15 mL YEME [0.3% (w/v) yeast extract, 0.5% (w/v) bacto-peptone, 0.3% (w/v) malt extact, 1.0% (w/v) glucose, 34% (w/v) sucrose] containing 1% maltose and 0.005 M $MgCl_2$] and incubated on a rotary shaker at 250 rpm for 24 hr at 28°C. Culture conditions and media used for preparing *S. lividans* TK24 cells for protoplasting were as described by Hopwood et al. (1985).

The *E. coli* strain used for most of this study was MV1193 (Vieira and Messing, 1987). This strain and the *E. coli* plasmid pUC119 were gifts from J. Vieira (Waksman

Institute of Microbiology, Rutgers University, Piscataway, NJ, USA) *E. coli* ER1447 was also used for the generation of unmethylated plasmids for digestion by methylation sensitive restriction enzymes. The indicator organism *E. coli* ESS was obtained from A. L. Demain (Department of Biology, Massachusetts Institute of Technology, Cambridge). The *E. coli* cells were grown and competent cells prepared according to Morrison (1979). For all other purposes, *E. coli* strains were grown on 2YT [1.6% (w/v) tryptone, 1.0% (w/v) yeast extract, 0.5% (w/v) NaCl] broth or agar plates supplemented with either 50 μg/mL of ampicillin or 100 μg/mL apramycin to maintain plasmids. All other *E. coli* maintenance procedures and culture conditions followed standard procedures (Sambrook et al., 1989; Vieira and Messing, 1987).

Staphylococcus aureus N2 was obtained from the Department of Microbiology, University of Alberta, Edmonton, Canada.

Bacillus sp. ATCC 27860 a sensitive indicator for alanyl clavam was used for bioassays. Spores were used to inoculate 10 mL of nutrient broth + 0.2% glucose which was incubated overnight on a 30°C shaker. Cells were then washed in one volume of 0.85% NaCl then resuspended in 5 mL of 0.85% NaCl. 1.2 mL of the 0.85% NaCl cell suspention was then used to inoculate 100 mL of Davis minimal medium plus glucose and L-methionine [7% (w/v) K_2HPO_4, 3.0% (w/v) KH_2PO_4, 0.5% (w/v) Na_3 citrate·$3H_2O$, 0.1% (w/v) $MgSO_4 \cdot 7H_2O$, 1.0% (w/v) $(NH_4)_2SO_4$, 15% agar, pH 7.0, with the addition of 10% (v/v) of 20% (w/v) glucose, plus 0.4% (v/v) of 50% (w/v) L-methionine for control plates after autoclaving].

The plasmid pBB5.3-E was provided by Randy Ritimer (University of Alberta, Edmonton, Alberta, Canada). It consists of pUC119 with a 2.1 kb *EcoRI-BglII* fragment of *S. clavuligerus* DNA containing *orf-2* within the multiple cloning site.

The plasmid pUC120Ap(*NcoI*) was provided by Ashish Paradkar (University of Alberta, Edmonton, Alberta, Canada). It carries an apramycin resistance gene (*apr*) in a

cassette flanked by *NcoI* sites which allows removal of the *apr* cassette from pUC120Ap(*NcoI*) as a *NcoI* fragment.

II.3 ISOLATION OF PLASMID DNA

Plasmids replicated in *S. lividans* and *S. clavuligerus* were isolated and purified according to the alkaline lysis method of Kieser et al. (1984) as described by Hopwood et al. (1985) for small and large scale preparations.

E. coli plasmids were isolated according to a modification of the method of Birnboim and Doly (1979) recorded in Sambrook et al. (1989). All plasmids were analyzed by agarose gel electrophoresis.

II.4 INTRODUCING DNA BY TRANSFORMATION

II.4.1 Transformation of *S. lividans* TK24

The procedure to make protoplasts of *S. lividans* TK24 was previously described in Hopwood et al. (1985). There were however, slight modifications to the procedure described for transformation. Instead of a large 50 mL centrifuge tube being used for the transformation procedure, a sterile 1.5 mL centrifuge tube containing a 100 µL aliquot of protoplasts (approx. 1 x 10^9 cells) was centrifuged in a Eppendorf Centrifuge 5415 C at 6000 rpm for 3 min. The supernatant was discarded and cells were resuspended in the residual liquid by gently tapping the tube. The volumes used during the transformation procedure were decreased by a factor of 5 for all the procedures compared to Hopwood et al., except the DNA which remained at a volume of 10-20 µl. After 24 hr of regeneration at 30°C, the plates were overlaid with 2.5 mL of soft nutrient agar containing thiostrepton to give a final concentration per plate of 5 µg/mL.

II.4.2 Protoplast formation and transformation of *S. clavuligerus*

The procedure used for making protoplasts from *S. clavuligerus* cells was based on the method outlined by Hopwood et al. (1985). Spores were used to inoculate 25 mL TSB + 1% maltose followed by incubation at 28°C and 250 rpm. After 48 hr incubation, 1 mL of this seed culture was transferred to 25 mL of TSB/YEME and incubated on a rotary

shaker at 250 rpm for 24 hr at 28°C. The mycelium was then harvested in a 50 mL centrifuge bottle at 12,000 x g for 10 min and washed twice in 10.3% sucrose. The mycelium was then resuspended in 4 mL of lysozyme buffer consisting of 1 mg/mL of lysozyme dissolved and filter sterilized in P-buffer [0.3 M sucrose, 25 mM MOPS, pH 7.2, 0.057 mM K_2SO_4, 48 mM $CaCl_2$, 0.59 µM $ZnCl_2$, 1.48 µM $FeCl_3$, 0.12 µM $CuCl_2$, 0.10 µM $MnCl_2$, 0.052 µM Na_2B_4O, and 1% (w/v) bovine serum albumin]. The sample was then incubated at 28°C with gentle shaking (50 rpm) for 20 min. To determine if the formation of protoplasts was complete, samples were visually scrutinized under a phase contrast microscope. If filamentous mycelia could still be detected the sample was triturated with a 5 mL pipette and incubated a further 10 min. When protoplasting was complete 5 mL of P-buffer was added to dilute the reaction, triturating as above. The cells were then filtered through sterile cotton wool filter by gravity to remove any unprotoplasted mycelium. The protoplasts were then centrifuged at 1000 x g for 7 minutes and washed once in 10 mL of P-buffer. The protoplasts were then dispensed into 1.5 mL centrifuge tubes at 1 x 10^9 protoplasts per tube. At this stage protoplasts may be frozen at -70°C and stored for later use.

The procedure for introducing plasmid DNA into *S. clavuligerus* is based on the method of Bailey and Winstanley (1986) with minor modifications. An aliquot of frozen protoplasts (10^9) was thawed quickly and washed with one mL of P-buffer. Cells were centrifuged in a microfuge at 3000 x g for 3 min and the pellet was gently resuspended in the remaining P-buffer after decanting. The cells were then heat pulsed at 43°C for 5 min. DNA (up to 20 µl) was added and immediately 100 µl of T-buffer [P-buffer that contained 25% polyethylene glycol 1000 (Koch Light)] was added and mixed with the cells and DNA by pipetting. One mL of P-buffer was then added to stop the reaction. Transformed cells were centrifuged as above and resuspended in 500 µl of P-buffer and plated onto modified R5B regeneration agar [10% (w/v) sucrose, 1.1% (w/v) sodium glutamate, 5.1% (w/v) $MgCl_2 6H_2O$, 0.1% (w/v) casamino acids, 0.005% (w/v) $MgSO_4 7H_2O$, 1% (w/v) starch,

0.57% (w/v) TES, pH 7.2; followed by the addition of 0.2% (v/v) trace elements (0.004% (w/v) $ZnCl_2$, 0.02% (w/v) $FeCl_3 6H_2O$, 0.001% (w/v) $CuCl_2 2H_2O$, 0.001% (w/v) $MnCl_2 4H_2O$, 0.001% (w/v) $Na_2B_4O_7 \cdot 10H_2O$, 0.001% (w/v) $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.5% (v/v) of 5M $CaCl_2 \cdot 2H_2O$, and 1.0% (v/v) of 0.5% (w/v) KH_2PO_4 after autoclaving]. Plates were incubated at 28°C for 48 hr before overlaying with 2.5 mL soft nutrient agar containing thiostrepton to give a final concentration per plate of 5 μg/mL.

II.4.3 Transformation of *E. coli*

The preparation and transformation of competent *E. coli* cells followed standard procedures outlined by Sambrook et al. (1989).

II.5 TECHNIQUES FOR DNA ANALYSIS

II.5.1 Restriction fragment analysis by agarose gel electrophoresis

DNA fragments were subjected to electrophoresis on 0.8% (w/v) agarose gels using a TEA buffer system (40 mM Tris-Acetate, pH 8.0, 1 mM EDTA). Appropriate molecular weight markers were either *Bst*EII or *Pst*I restriction fragments of λ phage DNA.

II.5.2 Preparation of Southern blots

The procedure followed for transferring separated DNA fragments from an agarose gel to a nylon membrane was an adaptation of the method of Southern (1975) as described by Sambrook et al. (1989). The gel of interest was soaked with gentle shaking in 0.25 N HCl for 30 min. DNA fragments were denatured by soaking in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 min. The gel was then neutralized in 3 M sodium acetate for 30 min (2 x 15 min). The gel was then placed on the transfer apparatus and DNA was then passed to a nylon membrane via capillary action using 20 x SSPE (3 M NaCl, 0.2 M $NaH_2PO_4 \cdot H_2O$, 0.02 M Na_2EDTA, pH 7.4) as the transfer solution. After overnight transfer, the filter was allowed to air dry before baking in an 80°C vacuum oven for 2h.

II.5.3 Generation of nick-translated ^{32}P labeled probes

The method for labeling a DNA fragment by nick-translation is based on the protocol described by Sambrook et al. (1989). DNA restriction fragments were labeled

with 10 μCi of [α-^{32}P]dCTP in a buffer containing: 50 mM Tris-HCl, pH 7.5, 10 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM dithiothreitol (DTT), 50 μg/mL BSA, 0.01 ng DNaseI, 0.03 mM dTTP, 0.03 mM dATP, 0.03 mM dGTP, and 1 unit of *E. coli* DNA polymerase I at 15°C for 1h. The labeled probe was separated from unincorporated [α-^{32}P]dCTP on a 2 mL Sephadex G-50 column.

II.5.4 Hybridization of Southern blots

Blots were hybridized using the procedure outlined in the Amersham Manual (1985) with minor alterations. Nylon filters were placed in a screw cap hybridization tube with prehybridization solution [6 x SSPE, 5 x Denhardt's solution (Denhardt, 1986), and 10 μg/mL denatured salmon sperm DNA]. Ten and 20 mL of prehybridization solution was used for the small and large roller tube respectively. The blot was then incubated at 65°C while rotating in a hybridization oven.

Hybridization of the desired probe was performed by replacing the prehybridization solution with hybridization solution (prehybridization solution with approx. 10 μCi of denatured ^{32}P-labeled probe). The tube was once again placed in the hybridization oven to continue incubation with rotation overnight at 65°C. The filter was then removed and washed with 2 x SSPE/0.1% SDS at room temperature for 10 min, followed by 1 x SSPE/0.1% SDS at 65°C for 15 min, and a final wash with 0.1 x SSPE/0.1% SDS at 65°C for 10 min. Filters were then wrapped in plastic wrap and exposed to Kodak X-OMAT AR film at -70°C.

II.6 CONSTRUCTION OF THE *orf-2* DISRUPTION VECTOR

II.6.1 Introduction of *Not*I-*Nco*I linker/adapter

To accommodate the insertion of the *Nco*flanked *apr* fragment into *orf-2* at the *Not*I site, a 14-mer oligonucleotide 5′-GGCCGACCATGGTC-3′was synthesized which can self-hybridyze to give a double-stranded fragment which contains a central *Nco*I restriction site, as well as four nucleotide long 5′ overhangs which are complementary to the sticky ends of *Not*I digested DNA. Since only the overhanging ends are complementary to the

*Not*I recognition sequence, insertion of the linker at a *Not*I site would introduce a *Nco*I site while altering the *Not*I site making it inactive. The synthetic oligonucleotide was annealed by diluting 1 µl of oligonucleotide (223.2 pmole/µl) to 20 µl with annealing buffer (Sambrook et al., 1989) and incubated at 75°C for 10 min, then allowed to cool slowly to room temperature. From the annealed oligonucleotide mixture, 0.5 µl (5.6 pmole) of annealed oligonucleotide was added to a 20 µl total volume ligation mixture containing 0.32 pmoles of *Not*I digested pBB5.3-E. The annealed oligonucleotide was ligated into *Not*I digested pBB5.3-E using standard procedures (Sambrook et al., 1989) and transformed into competent *E. coli* ER1447. Transformed cells were then plated onto 2YT + 100 µg/mL ampicillin plates and incubated overnight at 37°C. Twenty ampicillin resistant colonies were selected for screening of plasmids which have a *Nco*I restriction site. Each was inoculated into a roller tube containing 2 mL of 2YT + 100 µg/mL ampicillin and incubated at 37°C overnight. Plasmid was purified from each of the isolates and then each was digested with *Nco*I. Plasmid from one of the twenty isolates was linearized by digestion with *Nco*I. To confirm that the *Not*I-*Nco*I linker/adapter had correctly inserted into the *Not*I site of pBB5.3-E to form pKEO2*Nco*, plasmid from the isolates was also digested with *Not*I to show that pKEO2*Nco* no longer carries a *Not*I restriction site.

II.6.2 Insertion of the *apr* gene into *orf-2*

To insert an apramycin resistance gene into the coding sequence of *orf-2*, pKEO2*Nco* and pUC120Ap(*Nco*) were both digested with *Nco*I using standard protocols. The plasmid pUC120Ap(*Nco*) carries an apramycin resistance gene (*apr*) in which the terminal ends of the fragment have been modified to allow removal from pUC120Ap(*Nco*) as a *Nco*I fragment. The fragments from *Nco*I digested pUC120Ap(*Nco*) were then separated on an 0.8% agarose gel by electrophoresis. After examining the ethidium bromide stained gel under ultraviolet light, the 1.45 kb fragment corresponding to the *apr* gene was cut from the agarose. The DNA was then purified from the agarose using a Qiaquick™ purification kit as per the manufacturers instructions. The *apr* fragment was

then ligated into *Nco*I digested pKEO2*Nco*, followed by transformation into competent *E. coli* ER1447 cells. The transformed cells were incubated with 0.5 mL of 2YT at 37°C for 2 hours before plating onto 2YT plus 100 µg/mL apramycin. After overnight incubation at 37°C, an apramycin resistant colony was selected for plasmid purification, followed by restriction enzyme digestion of the purified plasmid to confirm that the *apr* fragment had inserted into pKEO2*Nco* to make pKEO2Ap.

II.6.3 Transfer of the *apr* disrupted *orf-2* gene to pIJ486

The fragment containing the *apr* disrupted *orf-2* gene was removed from pKEO2Ap by restriction enzyme digestion using *Eco*RI and *Hin*dIII. The 3.5 kb *Eco*RI-*Hin*dIII O2Ap fragment was separated from vector DNA by electrophoresis on a 0.8% agarose gel. The O2Ap fragment was cut from the gel and the DNA extracted from the agarose using a Qiaquick™ DNA purification kit as per the manufactures instructions. The *Eco*RI-*Hin*dIII O2Ap fragment was then ligated into similarly digested pIJ486, followed by transformation into protoplasts of *S. lividans* TK24. Transformants were chosen by positive selection for apramycin resistance. Plasmid was then purified from transformants and the plasmid scrutinized by restriction enzyme analysis to confirm the correct plasmid pIJO2Ap had been constructed.

II.7 CONSTRUCTION OF THE *S. CLAVULIGERUS Orf-2* MUTANT

The *Streptomyces* vector pIJO2Ap was passaged through *S. lividans* TK24 to increase the efficiency of transformation into *S. clavuligerus*. Purified pIJO2Ap was transformed into wild-type *S. clavuligerus* protoplasts using the standard protocol. Thiostrepton resistant transformants were then patched onto ISP-3 (5 µg/mL thiostrepton, 25 µg/mL apramycin). Transformants which were both thiostrepton and apramycin resistant were selected for transfer to ISP-3 for sporulation in the absence of antibiotic selection. Spores were harvested from the patches then used to inoculate ISP-3 plates to generate sufficient spores for further study. After sporulation, the spores were harvested using standard protocol, serially diluted in 0.1% peptone to 10^{-7}, and plated in duplicate on

ISP-3 master plates to allow for isolated colonies. Once the isolated colonies had sporulated, the master plates were replicated onto ISP-3, ISP-3 (5 μg/mL tsr), and ISP-3 (25 μg/mL apr) plates. Colonies that corresponded to apramycin resistant and thiostrepton sensitive mutants were selected for further study and confirmation of the disruption of *orf-2*.

II.8 ACETOHYDROXY ACID SYNTHETASE ASSAY

II.8.1 Preparation of a cell free extract

Cells were harvested by centrifugation in 250 mL bottles at 3000 x g at 5°C. All subsequent manipulations were done on ice or at 5°C. The pellet was then washed once in 100 mM potassium phosphate buffer pH 7.5 plus 10% (w/v) glycerol. After this stage pelleted cells were either used directly or stored at -70°C. The washed pellets were resuspended in 100 mM phosphate buffer pH 7.5 plus 10% (w/v) glycerol at 1 g of wet mycelia to 1 mL of buffer. The cell suspension was sonicated at maximum intensity for three 15 second bursts with 30 second cooling intervals. The broken cell suspension was centrifuged at 25,000 x g for 20 min, the supernatant was recovered and used as the crude enzyme extract. This method provided between 5 and 20 mg of protein per mL.

II.8.2 AHAS isozyme analysis

The assay used was based on that of De Felice et al.(1988), which is specific for the determination of relative amounts of isozymes I and III in *E. coli* K12.

Four different assay mixtures were used:

A: 40 mM sodium pyruvate, 170 μM thiamine pyrosphosphate chloride, 10 mM $MgSO_4$, 100 mM potassium phosphate buffer, pH 6.6

B: Same as A plus 25 μM FAD

C: Same as A, with 100 mM Tris-HCl buffer, pH 9.3, in the place of potassium phosphate buffer

D: Same as B with 100 mM Tris-HCl buffer, pH 9.3, in the place of potassium phosphate buffer

Controls contained the same components as A-D, except that pyruvate was omitted.

An aliquot of extract containing from 50 to 500 μg of protein was added to each assay mixture, for a total volume of 1 mL, in order to start the reaction. Reaction mixtures were incubated for 20 min at 28°C, the reaction was then stopped by adding 0.1 mL of 50% (v/v) H_2SO_4 and samples were kept at 28°C for 30 min. Acetolactate formed as the result of AHAS activity was assayed as acetoin using the colorometric assay of Westerfeld(1945). Coloring solutions (0.5% (w/v) creatine and 1% (w/v) α-naphthol in 5 N NaOH) were freshly prepared and 1 mL of each solution was added to each tube. After 30 min incubation at 28°C, samples were clarified with a brief centrifugation at room temperature and the absorbance at 520 nm was read using the mixtures without pyruvate as blanks. A standard curve was prepared using reaction mixtures that are identical to the blanks plus 20 to 200 nmol of acetoin. These samples were incubated, treated, and read as the assay samples after addition of extracts.

II.8.3 pH Optimum.

Optimum pH was determined using reaction mixture A, but containing either phosphate or Tris-HCl buffer with pH increments of 0.5. Phosphate buffer was used from pH 6.0 to 8.0 and Tris-HCl buffer from pH 7.0 to 9.5.

II.8.4 Standard AHAS assay

Standard reaction mixture for optimal reaction conditions contained: 40 mM sodium pyruvate, 170 μM thiamine pyrosphosphate chloride,10 mM $MgSO_4$, 100 mM potassium phosphate buffer pH 7.5. Reaction conditions were as for the AHAS isozyme analysis .

II.8.5 *Feedback inhibition.*

Valine and isoleucine inhibition studies were done by adding valine and/or isoleucine to the standard reaction mixture to a maximum concentration of 10 mM. Enzyme activity in these reactions was then compared to samples with no amino acid added and expressed as percent inhibition by valine and/or isoleucine.

II.9 β-LACTAM BIOASSAYS

Bioassays were conducted by the agar plate diffusion method with the appropriate indicator organism seeded into TSBS agar. Wild-type and *orf-2* mutant were inoculated into 100ml shake flasks containing either starch-asparagine or soy media and incubated at 28°C and 250 rpm. At 48 and 72 hours the cultures were harvested and a sample of the supernatant collected. Culture supernatants were centrifuged to remove any mycelia or particulate matter and then a 25 μL sample was applied to a filter disk on the surface of TSBS inoculated with the appropriate indicator organism.

II.9.1 β-Lactam antibiotic assay

Total antibiotics were determined with *E. coli* ESS as the indicator organism (Aharonowitz and Demain, 1978). After 24 hours, zones of inhibition were measured in millimeters as a function of the amount of β-lactam antibiotics in the culture supernatant.

II.9.2 Clavulanic acid bioassay

β-Lactamase inhibitory activity was determined with *Staphylococcus aureus* N2 as the indicator organism in the presence of 1 μg/mL penicillin G. Control plates lacked penicillin G. After 24 hours, zones of inhibition were measured in millimeters as a function of the amount of clavulanic acid in the culture supernatant.

II.9.3 Alanylclavam bioassay

The antimetabolite activity of 2-alanyl-clavam was determined with *Bacillus sp.* ATCC 27860 as the indicator organism (Pruess and Kellett, 1983). Samples of culture supernatant were applied to filter disks on the surface of Davis minimal media (plus glucose) inoculated with *Bacillus sp.* ATCC 27860. Control plates contained L-methionine (200 μg/mL) to counter act the antimetabolite activity of 2-alanyl-clavam. Zones of inhibition were measured after overnight growth at 30°C.

II.9.4 HPLC Assay of Clavams

The high-pressure liquid chromatography (HPLC) assay has been described previously (Foulstone and Reading, 1982). Analysis was performed with a model M-45

pump, model 712 WISP automated sample injector, and model 480 variable-wavelength UV detector (set at 312 nm), all from Millipore Waters (Mississauga, Ontario, Canada). Culture supernatants were centrifuged to remove particulate matter, then a 100 μL sample was mixed with imidazole reagent (25 μL) and incubated at room temperature for 15 min to form imidazole-derivatized clavams (Bird et al., 1982). A 50 μL sample of the derivatized supernatant was then analyzed on a reverse-phase column (Bondapak-C18) with an isocratic buffer system consisting of 0.1 M KH_2PO_4-6% methanol, pH3.2 (adjusted with H_3PO_4).

III. Results:

With the unexpected identification of the putative clavulanic acid biosynthetic gene cluster by Jensen et al., research within the group has been focused on determining the function of the ORFs surrounding known clavulanic acid biosynthetic genes. The first open reading frame (*orf-2*) downstream of the rightward end of the cephamycin gene cluster was a likely candidate for study due to its close proximity to clavulanic acid and cephamycin biosynthetic genes. The goal of this research was to determine, via gene disruption, if *orf-2* is involved in clavulanic acid and/or cephamycin biosynthesis, or some alternate scheme.

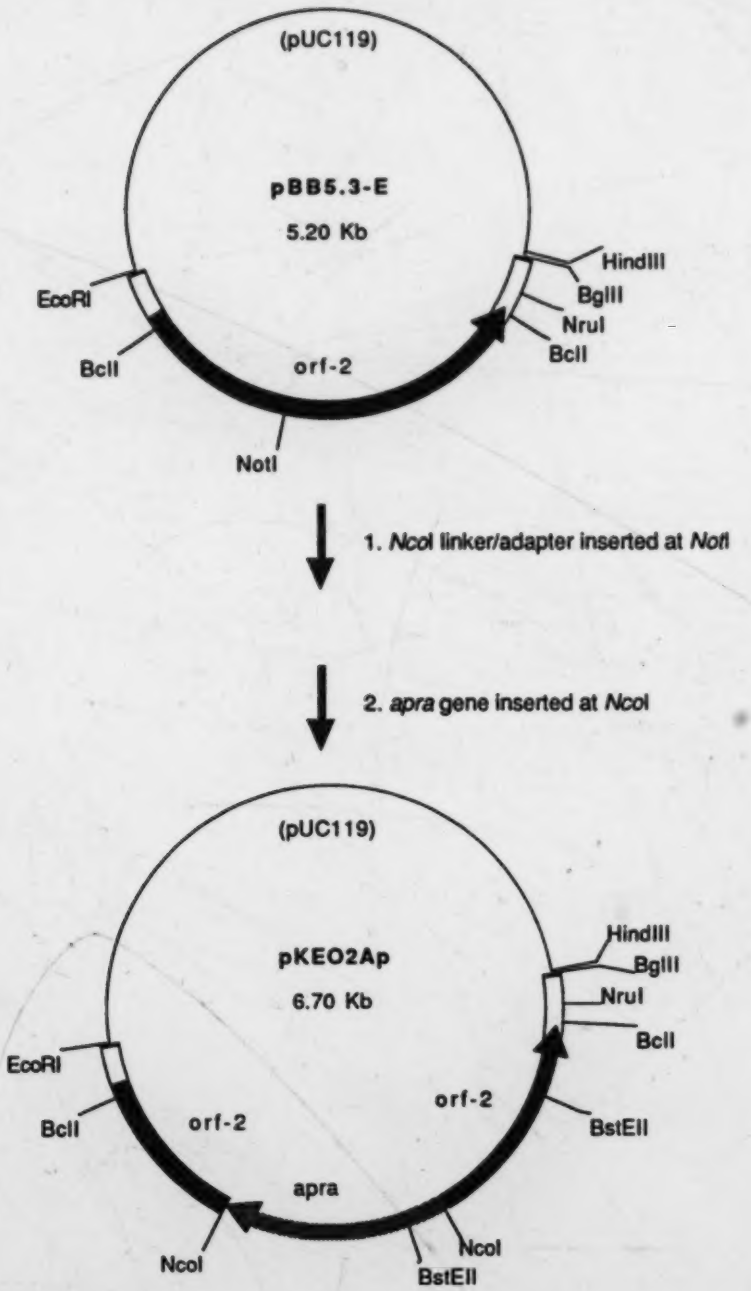
III.1 DISRUPTION OF *ORF-2*.

The specific inactivation of *orf-2* was accomplished by the insertion of a non-homologous DNA sequence within the reading frame of a cloned copy of *orf-2*. In this case, the insertion sequence was an apramycin resistance gene which provides a convenient marker to follow the inactivated copy of *orf-2*. The disrupted copy of *orf-2* was inserted into a segregationally unstable *Streptomyces* plasmid vector and transformed into wild-type *S. clavuligerus* to allow homologous recombination and crossing-over of the disrupted *orf-2* plasmid copy with the wild-type *orf-2* to occur.

III.1.1 Insertion of a *NotI/NcoI* linker/adapter

To determine the function of *orf-2*, a 2.1-kb *Eco*RI-*Bgl* II fragment containing *orf-2* was cloned into the multiple cloning site of pUC119 resulting in the plasmid pBB5.3-E (R. Rittimer, unpublished) (Figure 8). pBB5.3-E was used for the further manipulation and analysis of *orf-2*. To disrupt the reading frame of the cloned copy of *orf-2* on pBB5.3-E, the *Not*I restriction endonuclease site was chosen for the insertion of a fragment of DNA encoding apramycin resistance (*apr*). The *Not*I site was chosen since it is centrally located within the *orf-2* fragment which allows sufficient *orf-2* sequence both up and downstream of the *Not*I site to allow efficient homologous recombination with the wild-type copy of *orf-2*. The apramycin resistance gene was selected for insertion within the

Figure 8: Construction of the *orf-2* disruption plasmid. The plasmid pBB5.3-E is a pUC119 based vector that contains *orf-2* within its multiple cloning site as a *Eco*RI-*Bgl*II fragment. pKEO2Ap was created by converting the *Not*I site of pBB5.3-E to a *Nco*I site by ligating a *Not*I/*Nco*I linker/adapter into *Not*I digested pBB5.3-E, followed by the insertion of a *Nco*I flanked apramycin resistance gene (*apra*) into the newly created *Nco*I site. The boxed regions of the plasmids contain DNA from *S. clavuligerus*.


(pUC119)
pBB5.3-E
5.20 Kb
EcoRI
BclI
orf-2
NotI
HindIII
BglII
NruI
BclI
1. NcoI linker/adapter inserted at NotI
2. apra gene inserted at NcoI
(pUC119)
pKEO2Ap
6.70 Kb
EcoRI
BclI
orf-2
apra
NcoI
BstEII
orf-2
BstEII
HindIII
BglII
NruI
BclI

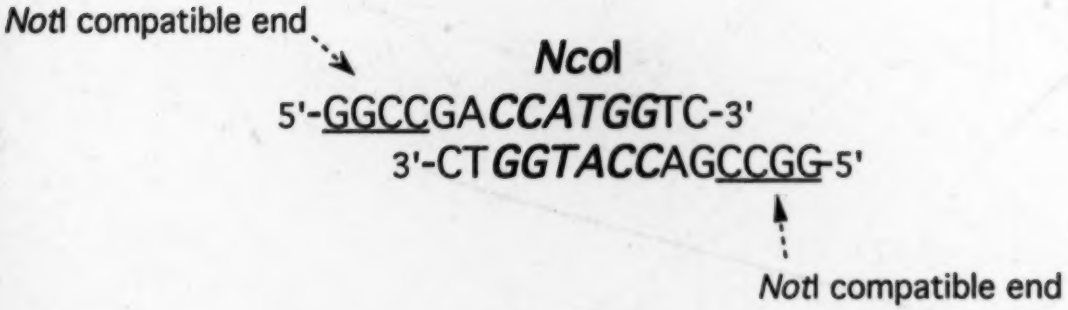
coding sequence of *orf-2*, since besides disrupting the coding sequence of *orf-2*, it provides a useful marker (apramycin resistance) selectable in both *E. coli* and *Streptomyces*. The *apr* gene was readily available as a 1.45-kb *NcoI* fragment from pUC120Ap (see section II.2 of Materials).

To accommodate the insertion of the *Nco*I flanked *apr* fragment into *orf-2* at the *Not*I site, a 14-bp oligonucleotide linker/adapter was made which contains a central *Nco*I restriction site, as well as four base pair long 5′ overhangs which are complementary to the sticky ends of *Not*I digested DNA (Figure 9). Since only the overhanging ends are complementary to the *Not*I recognition sequence, insertion of the linker at a *Not*I site would insert a *Nco*I site while altering the *Not*I site making it inactive. The *Nco*I linker was ligated into pBB5.3-E and transformed into competent cells of *E. coli* MV1193. Twenty ampicillin resistant transformants were selected and examined for plasmids which contain the *Nco*I linker. Plasmid from each transformant was digested separately with *Nco*I and *Not*I to screen for plasmids which have the *Nco*I linker inserted at the *Not*I site. *Nco*I is not present in pBB5.3-E, therefore only plasmids that contain the *Nco*I linker would be linearized by *Nco*I. Since insertion of the linker would destroy the *Not*I site, only plasmids containing the linker would not be cut by *Not*I. Of the twenty transformants selected, one plasmid had the correct restriction pattern, which corresponds to the *Nco*I linker inserted into the *Not*I site of pBB5.3-E resulting in the plasmid pKEO2*Nco*.

III.1.2 Ligation of the *apr* cassette into pKEO2*Nco*

The *NcoI* flanked apramycin resistance gene cassette carried on pUC120Ap(*Nco*) was liberated by digestion with *NcoI*, followed by separation of the fragments by gel electrophoresis. The 1.45-kb *apr* fragment was cut from the gel and purified using a Qiaquick DNA purification kit as per the manufacturer's instructions. The cassette was then ligated into the *Nco* I site of pKEO2*Nco* resulting in pKEO2Ap. The ligation mixture was then transformed into competent cells of *E. coli* MV1193. Transformants containing pKEO2Ap were identified by positive selection for apramycin resistance. The

Figure 9: Linker/adapter oligonucleotide used to introduce a *Nco*I site into pBB5.3-E.


NotI compatible end
NcoI
5'-GGCCGACCATGGTC-3'
3'-CTGGTACCAGCCGG-5'
NotI compatible end

disruption of *orf-2* with the apramycin resistance gene was confirmed by further restriction digest analysis. The orientation of the *apr* cassette was determined by restriction digestion of pKEO2Ap with *Bst*EII. *Bst*EII cuts near to the 5′ end of the *apr* cassette and within the 3′ portion of *orf-2* (see Figure 8). Digestion of pKEO2Ap resulted in two fragments, 5.6 kb and 1.1 kb, indicating that *apr* is in the opposite orientation to that of *orf-2*. If the *apr* cassette was in the same orientation to that of *orf-2*, *Bst*EII digestion would have resulted in fragments of 4.7 kb and 2.0 kb.

III.1.3 Ligation of O2Ap into pIJ486 and transformation into *S. lividans*

The apramycin disrupted *orf-2* (O2Ap) was removed from pKEO2Ap by digestion with *Eco*RI and *Hin*dIII. *Eco*RI is on the left hand end of the O2Ap insert, while *Hin*dIII is just outside the right-hand end of the insert and in the remainder of the multiple cloning site of pUC119. O2Ap was then ligated into the multiple cloning site of similarly digested pIJ486 for transformation into *Streptomyces* spp. Direct transformation to *S. clavuligerus* with non-native DNA results in very low transformation frequencies due to restriction modification by *S. clavuligerus*. To circumvent this problem, plasmids isolated from *E. coli* are shuttled through *S. lividans* due to its higher frequencies of transformation. Resulting plasmid DNA, since it comes from a related *Streptomyces* species can be transformed into *S. clavuligerus* with greater efficiency. Nine apramycin-resistant *S. lividans* transformants arose after 48 hours growth. Plasmid was purified from the nine transformants and analysed by restriction analysis to confirm that the correct plasmid was present.

III.1.4 Transformation of *S. clavuligerus* with pIJO2Ap

Purified pIJO2Ap from *S. lividans* was then transformed into wild-type *S. clavuligerus* protoplasts. Approximately 40 thiostrepton-resistant transformants arose from the transformation. Twelve of these transformants were then patched onto ISP-3 (5 μg/mL thiostrepton, 25 μg/mL apramycin) and eleven of the twelve transformants grew. Four of these transformants were selected for transfer to ISP-3 medium (sporulation medium) for

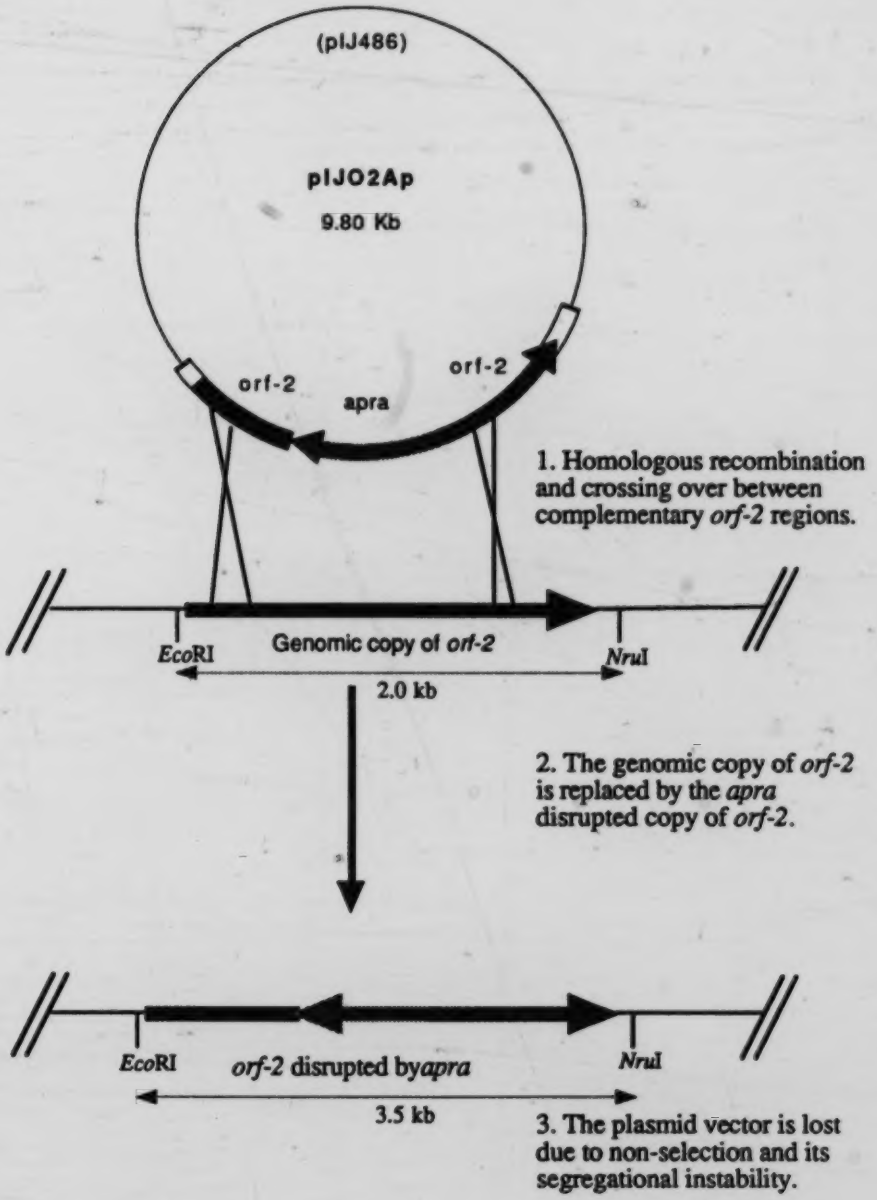
sporulation in the absence of antibiotic selection. Sporulation stimulates the loss of unselected plasmid, therefore plasmid encoded markers that have not inserted within the genome via homologous recombination and crossing-over are lost. After sporulation, the spores were harvested, serially diluted, and plated in duplicate on ISP-3 master plates to allow for isolated colonies. Once the isolated colonies had sporulated, the master plates were replicated onto ISP-3 (5 µg/mL tsr), ISP-3 (25 µg/mL apr) and finally ISP-3 plates to ensure that efficient replica plating had been carried out. Replica plating onto ISP-3 (5 µg/mL tsr) and ISP-3 (25 µg/mL apr) determined which of the isolated colonies on the master plates had arisen from a double cross-over event where the plasmid-derived, disrupted copy of *orf-2* replaces the wild-type *orf-2* followed by the loss of the plasmid (Figure 10). If such an event was to occur, the resulting mutants would only grow on ISP-3 (25 µg/mL apr), while not on ISP3 (5 µg/mL tsr), since the plasmid derived *orf-2* gene disrupted by the apramycin resistance gene would have replaced the wild-type *orf-2*. The remainder of the plasmid vector would be lost due to non-selection of the segregationally unstable pIJ486 based plasmid. Three colonies that were apramycin resistant and thiostrepton sensitive were selected for further study and confirmation of the disruption of *orf-2*.

III.2 SOUTHERN ANALYSIS

To confirm that the disrupted copy of *orf-2* has replaced the wild-type *orf-2*, genomic DNA from the three apramycin-resistant thiostrepton-sensitive transformants was examined by Southern analysis. If a complete replacement of *orf-2* with the disrupted *orf-2* from pIJO2Ap occurs, the wild-type *orf-2* is exchanged with the disrupted *orf-2* encoded on the plasmid pIJO2Ap. In the absence of thiostrepton selection the plasmid is then lost leaving only the genomically encoded *orf-2* disrupted by the apramycin resistance gene.

The disrupted *orf-2* may have also inserted as the result of a single cross over event. This would result in the wild-type *orf-2* and the disrupted *orf-2*, plus vector

Figure 10: Model of the homologous recombination event where the wild-type *orf-2* is replaced by the *apra* disrupted copy from pIJO2Ap.


(pIJ486)
pIJO2Ap
9.80 Kb
orf-2
apra
orf-2
1. Homologous recombination and crossing over between complementary orf-2 regions.
EcoRI
Genomic copy of orf-2
NruI
2.0 kb
2. The genomic copy of orf-2 is replaced by the apra disrupted copy of orf-2.
EcoRI
orf-2 disrupted byapra
NruI
3.5 kb
3. The plasmid vector is lost due to non-selection and its segregational instability.

sequences, would be contained within the genomic DNA. Thiostrepton sensitivity and apramycin resistance of the mutants suggests that the plasmid derived thiostrepton resistance gene is not present. However, Southern analysis was required to confirm that only the double cross over mutants have been isolated.

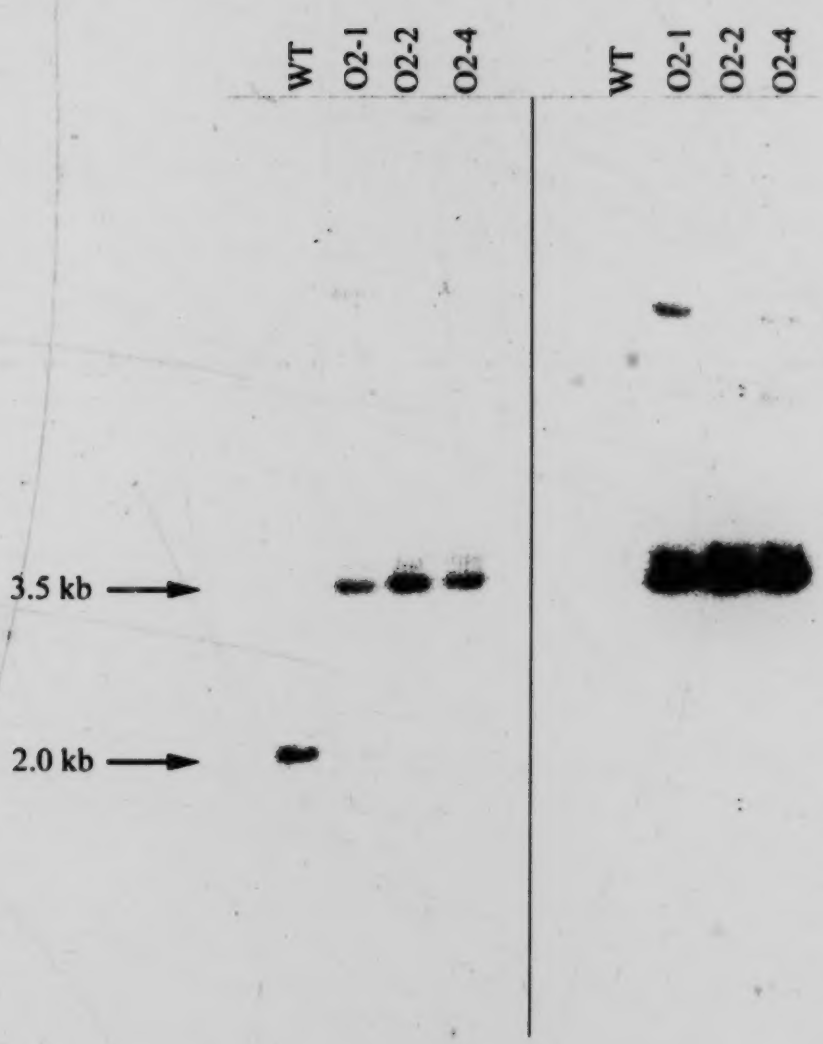
To confirm that the disrupted copy of *orf-2* was replaced the wild-type *orf-2*, genomic DNA from the three apramycin resistant transformants, as well as wild-type DNA, was digested with *Eco*RI and *Nru*I and examined by Southern blot analysis. If *orf-2* was replaced by the *apr* disrupted *orf-2*, an ^{32}P-labeled *orf-2* probe consisting of a 1.65 kb *Bcl*I fragment from pBB5.3-E (see Figure 8) should hybridize to a 3.5-kb *Eco*RI-*Nru*I fragment, while in the wild-type, the *orf-2* probe binds to a 2.0-kb *Eco*RI-*Nru*I fragment (Figure 10). As a confirmation, identical blots could be probed with a ^{32}P-labeled *apr* fragment. The *apr* fragment should hybridyze to the same 3.5-kb *Eco*RI-*Nru*I fragment in the *orf-2* disrupted mutants, while it should not hybridize to any DNA from the wild-type, since the *apr* gene should not be present.

Duplicate samples of total DNA from wild-type and *orf-2* mutants were digested with *Eco*RI and *Nru*I and then separated by agarose gel electrophoresis, followed by transfer to a nylon membrane. The membranes were then probed with either a ^{32}P-labeled 1.65-kb *Bcl*I-*Bcl*I *orf-2* fragment from pBB5.3-E, or a 1.45-kb *apr* fragment from pUC120Ap. After exposure to x-ray film, it was shown that the *orf-2* probe hybridized to a 3.5-kb fragment in the three mutants and a 2.0-kb fragment in the wild-type (Figure 11a). When an identical blot was probed with the *apr* fragment, the same 3.5-kb fragment was identified, while no hybridization was seen in the wild-type lane (Figure11b). These results confirm that the wild-type *orf-2* had been replaced by a disruptionally inactivated copy of *orf-2*.

III.3 ANALYSIS OF THE *S. clavuligerus ORF-2* MUTANTS.

Southern analysis has shown that the wild-type *orf-2* has been replaced by a disruptionally inactivated copy of *orf-2*. Our assumption is that disruptional inactivation of

Figure 11: Southern blot analysis of genomic DNA from wild-type *S. clavuligerus* (WT) and three *orf-2* mutants (O2-1, O2-2 and O2-4) disrupted with *apra*. Genomic DNA was digested with *Eco*RI and *Nru*I and separated on two identical 0.8% agarose gels. After transfer to a nylon membrane, blot (A) was probed with a 1.65-kb *Bcl*I-*Bcl*I *orf-2* fragment, while blot (B) was probed with a 1.45-kb *apr* fragment.


WT
O2-1
O2-2
O2-4
WT
O2-1
O2-2
O2-4
3.5 kb
2.0 kb

orf-2 would cause a deficiency in *orf-2* activity. Therefore, the phenotype of the *orf-2* mutant can be compared to wild-type *S. clavuligerus* in order to determine the function of *orf-2*. It is known from preliminary evidence that the predicted protein sequence of *orf-2* resembles AHAS enzymes and that *orf-2* is closely linked to both clavulanic acid and cephamycin biosynthetic genes. Therefore analysis of the mutant for altered AHAS activity, clavulanic acid production, and cephamycin production is a logical starting point for examining the *orf-2* muntants. If *orf-2* is an AHAS enzyme, AHAS activity and possibly cephamycin production would be altered. On the other hand, if *orf-2* is involved in clavulanic acid production, there may be a disruption in the biosynthetic pathway to clavulanic acid which would result in altered clavulanic acid production.

III.3.1 AHAS Activity

To examine the the *orf-2* mutant for altered AHAS enzyme activity, wild-type enzyme activity was first characterized. A seed culture was first prepared by inoculating spores into 25 mL TSBS and incubating for 48 hr with shaking at 28°C. The seed culture was then used to inoculate 100 mL SA cultures which were also incubated at 28°C with shaking. After 48 hr the cultures were harvested and a crude enzyme extract was prepared from sonicated mycelium and then examined for AHAS activity. It was our hypothesis that if *orf-2* encodes a second AHAS enzyme, it may have different pH optimum and cofactor requirements from the primary AHAS. This hypothesis seems plausible since we envisioned *orf-2* to encode an AHAS-specific for secondary metabolism where physiological conditions have changed within the cell and the primary AHAS may no longer be effective or additional activity is needed. AHAS isozymes from species, such as the enteric bacteria, which have been characterized to have more than one AHAS isozyme, have different pH optimum and cofactor requirements. *S. clavuligerus* AHAS activity was examined for correlation to the activity of either or both of the two AHAS isozymes in *E.coli* K12. Isozyme I from *E.coli* retains 50% activity at pH 6.6 and is inactive at pH 9.3. As well, its activity is reduced by 75% in the absence of FAD. Isozyme III on the other

hand retains 50% activity at pH 9.3 and does not need added FAD as a cofactor. Therefore, if *S. clavuligerus* crude enzyme extract is assayed at these conditions, the AHAS enzyme activity in *S. clavuligerus* could be analysed for evidence that more than one AHAS is present with different conditions for optimum activity.

The crude cell extract from *S. clavuligerus* was assayed for AHAS activity at pH 6.6 and 9.3, with and without FAD. AHAS activity was unchanged in the presence of FAD at either pH over a range of enzyme concentrations (Figure 12), which suggests that *S. clavuligerus* does not have an AHAS that resembles isozyme I of enteric bacteria. If AHAS activity was similar to that of isozyme I from *E. coli*, a decreased amount of AHAS activity would be expected at pH 6.6 when FAD was omitted with little activity at pH 9.3.

The effect of pH on AHAS activity is shown in Figure 13. Optimum pH was found to be pH 7.5. It was also noted that enzyme activity was greater in potassium phosphate buffer at pH 7.5 than Tris-HCl buffer at pH 7.5. Further assays were done using potassium phosphate buffer pH 7.5. Since there is only a single peak of enzyme activity, there is no evidence of multiple forms of the enzyme with different pH optima. This correlates well with *Corynebacterium glutamicum*, a close relative of *Streptomyces* spp., and *Streptomyces avermitilis*, both of which have only one AHAS enzyme.

Feedback inhibition on *S. clavuligerus* AHAS was investigated to determine how it correlates with feedback inhibition of AHAS enzymes from other species. Inhibition is examined by adding valine and/or isoleucine to the standard AHAS reaction mixture and activity is compared to uninhibited *S. clavuligerus* AHAS activity. The crude enzyme from *S. clavuligerus* was inhibited, but only to a maximum of 10% inhibition with 10 mM valine or isoleucine. Enteric isozymes I and III were inhibited 90% with 1 mM valine and 45% with 1 mM isoleucine (Eggeling et al., 1987), while AHAS activity from *S. avermitilis* and *C. glutamicum* was only partially inhibited by branched chain amino acids to a maximum of 50%. These results were, however, for crude enzyme preparations, not purified enzymes. There are many contaminating factors in the crude extracts that might affect

Figure 12: Effect of pH and flavin adenine dinucleotide (FAD) on AHAS activity. A crude cell extract was prepared from *S.clavuligerus* and assayed for AHAS activity at pH 6.6 and 9.3, with and without FAD.

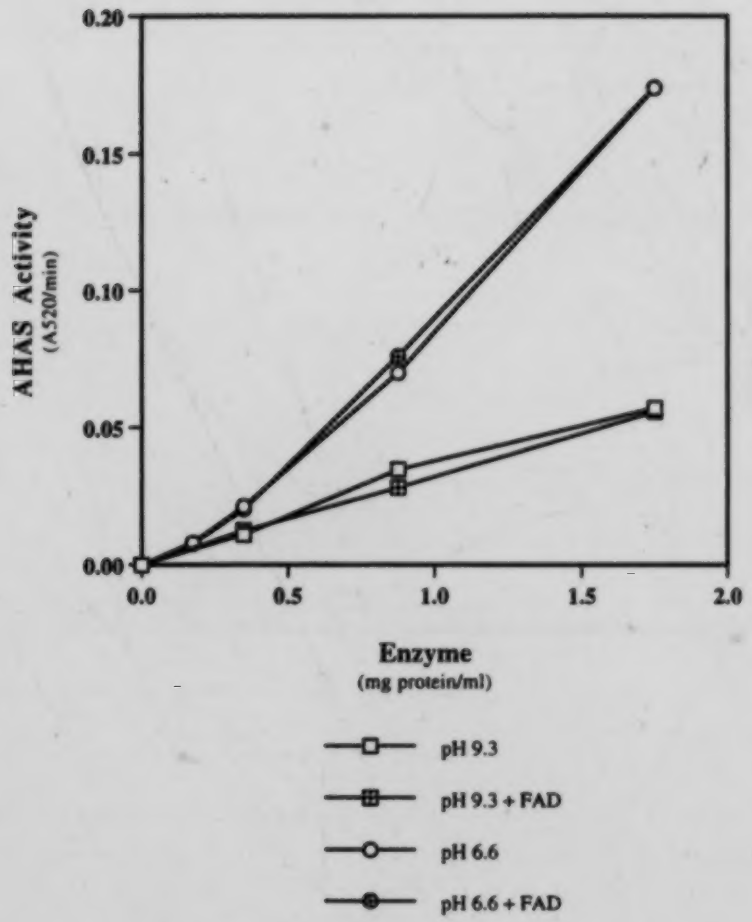
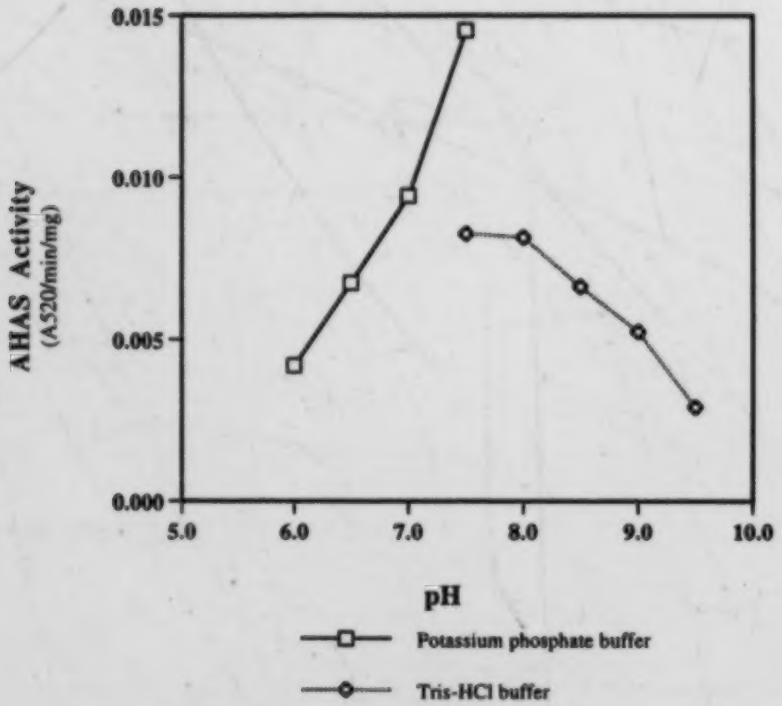

0.20
0.15
0.10
0.05
0.00
AHAS Activity
(A520/min)
0.0
0.5
1.0
1.5
2.0
Enzyme
(mg protein/ml)
pH 9.3
pH 9.3 + FAD
pH 6.6
pH 6.6 + FAD

Figure 13: Effect of pH on AHAS activity. A crude cell extract was prepared from *S.clavuligerus* and assayed for AHAS activity at varying pH with increments of 0.5. For pH 6-7.5 100 mM K^+ Phosphate buffer was used,100 mM Tris-HCl buffer was used for pH 7.5-9.5.


AHAS Activity
(A520/min/mg)
0.015
0.010
0.005
0.000
5.0
6.0
7.0
8.0
9.0
10.0
pH
Potassium phosphate buffer
Tris-HCl buffer

inhibition levels, such as free cytoplasmic amino acids contained in the enzyme extract. Therefore, inhibition characteristics of purified AHAS enzyme may differ greatly.

With wild-type AHAS activity characterized as having a only a single recognizable enzyme activity that remains constant over the growth period, we can use the standard AHAS assay to examine the *orf-2* mutant for altered AHAS activity. Mycelium from SA grown cultures of the wild-type and the *orf-2* mutant were harvested after 24, 48 and 72 hours growth and a crude enzyme extract prepared. Specific AHAS activity of the *orf-2* mutants was found to be essentially identical to the wild-type levels (Figure 14), indicating that the disruption of *orf-2* did not affect AHAS activity.

III.3.2 β-lactam antibiotic bioassay

To further examine the hypothesis that *orf-2* encodes a secondary AHAS specific to increasing levels of valine for incorporation into ACV and ultimately cephamycin production, culture supernatants were analysed for antibiotic (cephamycin) using a simple bioassay. *E. coli* (ESS) which is super-sensitive to β-lactam antibiotics was used as the indicator organism. Molten TSBS agar was inoculated with *E. coli* (ESS) and then poured into plates. After the agar solidified, filter disks were set on the surface of the agar, and a sample of culture supernatant was applied to the filter disks. After sufficient incubation (overnight at 37°C) to allow growth, levels of antibiotic were measured as a function of inhibition of the *E. coli* indicator organism by antibiotic contained in the culture supernatant. Supernatant from wild-type and *orf-2* mutant from SA or soy cultures caused equal sized zones of inhibition (Table 3). Therefore, we can conclude that *orf-2* is not involved in the production of cephamycin.

III.3.3 Clavulanic acid bioassay

If *orf-2* is not involved in cephamycin biosynthesis, it may be involved in clavulanic acid biosynthesis. It seems plausible that, since *orf-2* is located at the junction of the cephamycin and clavulanic acid gene clusters, *orf-2* is just as likely to be involved in

Figure 14: AHAS activity of wild-type *S. clavuligerus* and *orf-2* mutant at 24, 48 and 72 hours. Mycelium from SA cultures was harvested and a crude enzyme prepared.

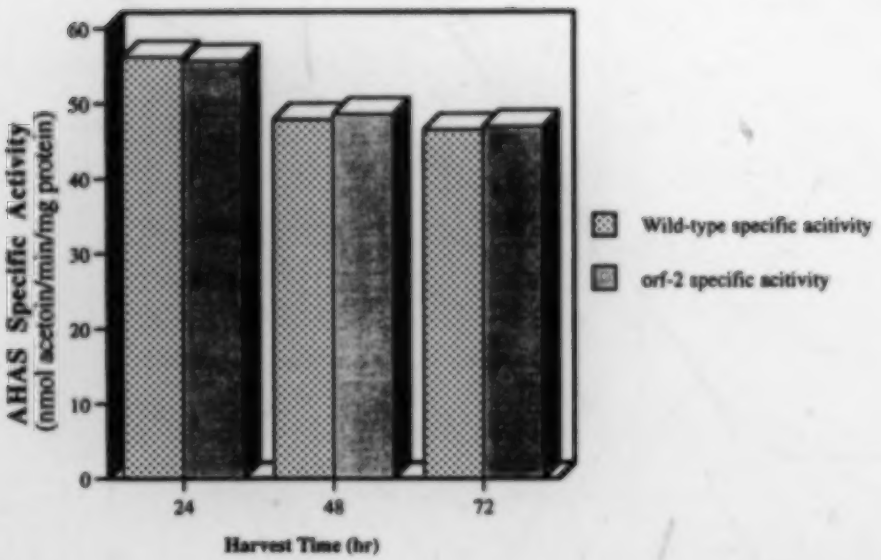

AHAS Specific Activity
(nmol acetoin/min/mg protein)
60
50
40
30
20
10
0
24
48
72
Harvest Time (hr)
Wild-type specific acitivity
orf-2 specific acitivity

Table 3: Cephamycin production by wild-type and *orf-2* mutant *Streptomyces clavuligerus*.

Growth[a] medium	Zone of Inhibition (mm)[b]	
	Wild-type	*orf-2* mutant
SA	31	30
Soy	34	35

a) Cultures were grown 72 hours in soy medium or SA medium before bioassay for antibiotic production.
b) 25 µl of culture supernatants were applied to filter paper disks on TSBS medium inoculated with *E. coli* ESS. Zones were measured after overnight incubation at 37°C.

clavulanic acid biosynthesis. Levels of clavulanic acid produced by the *orf-2* mutant were assayed and compared to the wild-type levels of clavulanic acid production.

Wild-type and *orf-2* mutant were inoculated from seed cultures into 100 mL shake flasks containing either SA or soy media and incubated at 28°C and 250 rpm. After 72 hours the cultures were harvested and a samples of the supernatants collected for clavulanic acid assays. Culture supernatants were centrifuged to remove any mycelia or particulate matter and then a sample was applied to a filter disk on the surface of TSBS medium containing 1 µg/mL penicillin G inoculated with *Staphylococcus aureus* N2. Duplicate samples were also applied to TSB agar lacking penicillin G to account for background levels of inhibition caused by antibiotic contained within the supernatants. After overnight incubation of the bioasssay plates, zones of inhibition were used to determine clavulanic acid concentrations (Table 4). The supernatant from *orf-2* SA cultures did not result in zones of inhibition, indicating that the *orf-2* mutant no longer has the ability to produce clavulanic acid when grown on SA medium. Supernatant from soy medium cultures showed slightly different results, a faint zone of inhibition 1-2 mm larger than the filter disk was seen for the 72 hr culture sample, however, control plates which lacked penicillin G also showed similar zone for both wild-type and *orf-2* mutant when grown on soy media.

III.3.4 Clavulanic acid HPLC assay

To further characterize clavam production by the *orf-2* mutant, supernatant was treated with imidazole and analysed by reverse phase HPLC with detection at 312 nm for clavam-imidazole derivatives. Samples without imidazole derivatization were also analysed by reverse phase HPLC to identify background peaks. Figure 15 shows the HPLC chromatograms of imidazole-derivatized compounds from wild-type and *orf-2* mutant cultures grown on SA medium with the background peaks subtracted. A large peak corresponding to clavulanic acid (8.79 min) is seen in the trace corresponding to the wild-type SA culture (Figure 15a), while no corresponding peak for clavulanic acid could be detected in the sample from the *orf-2* SA culture (Figure 15b). It can also be noted that

Table 4: Clavulanic acid production by wild-type and *orf-2* mutant *Streptomyces clavuligerus*.

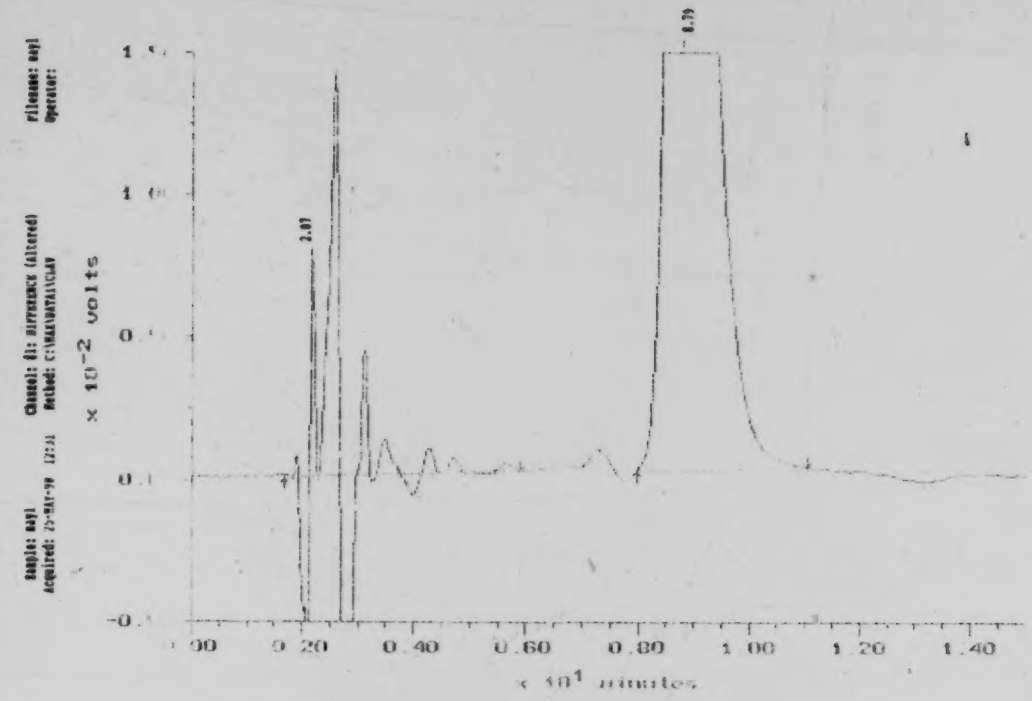
Growth[a] medium	Zone of Inhibition (mm)[b]	
	Wild-type	*orf-2* mutant
SA	31	0
Soy	37 (8)	8 (8)

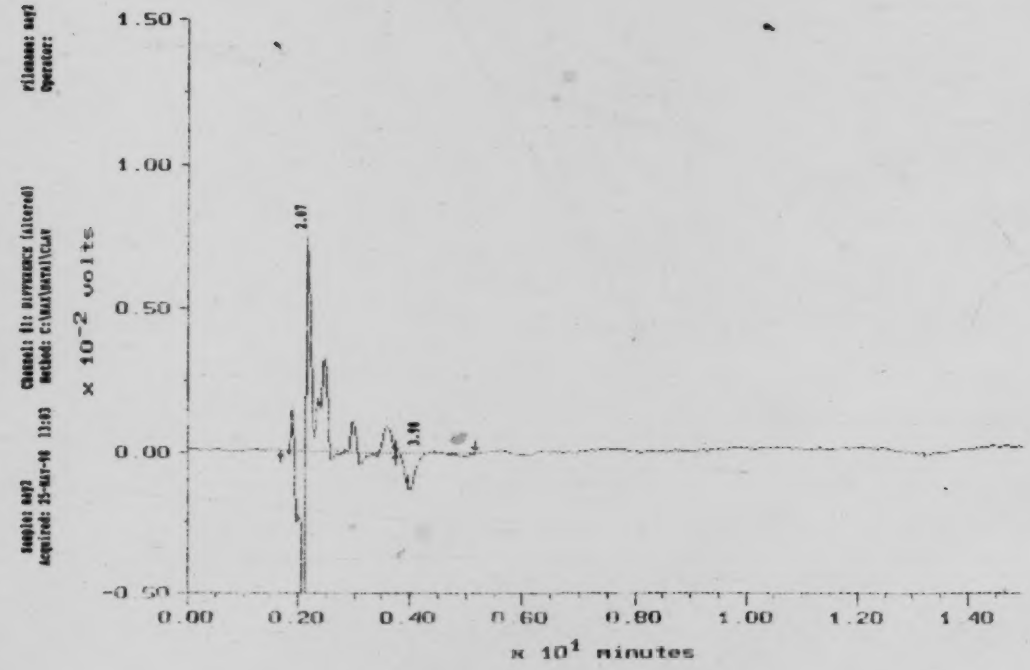
a) Cultures were grown 72 hours in soy medium or SA medium before bioassay for clavulanic acid production.

b) 25 µl of culture supernatants were applied to filter paper disks on TSBS medium (1 µg/ml penicillin G) inoculated with *Staphylococcus aureus* N2. Zones were measured after overnight incubation at 28°C.

c) Numbers in brackets indicate zones of inhibition on control plates which did not contain penicillin G.

Figure 15: Clavulanic acid and clavam metabolite production by wild-type and *orf-2* mutants of *S. clavuligerus* on SA medium - HPLC analysis. A) Wild-type. B) *orf-2* mutant. 100 µl of culture supernatant was mixed with imidazole reagent (25 µl) and incubated at room temperature for 15 min to form imidazole-derivatized clavams. A 50 µL sample of the derivatized supernatant was then analyzed on a reverse-phase column (Bondapak-C18) with an isocratic buffer system consisting of 0.1 M KH_2PO_4-6% methanol, pH3.2 with detection at 312 nm.


x 10^-2 volts
x 10^1 minutes
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40


1.50
1.00
0.50
0.00
-0.50
x 10^-2 volts
x 10^1 minutes
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40

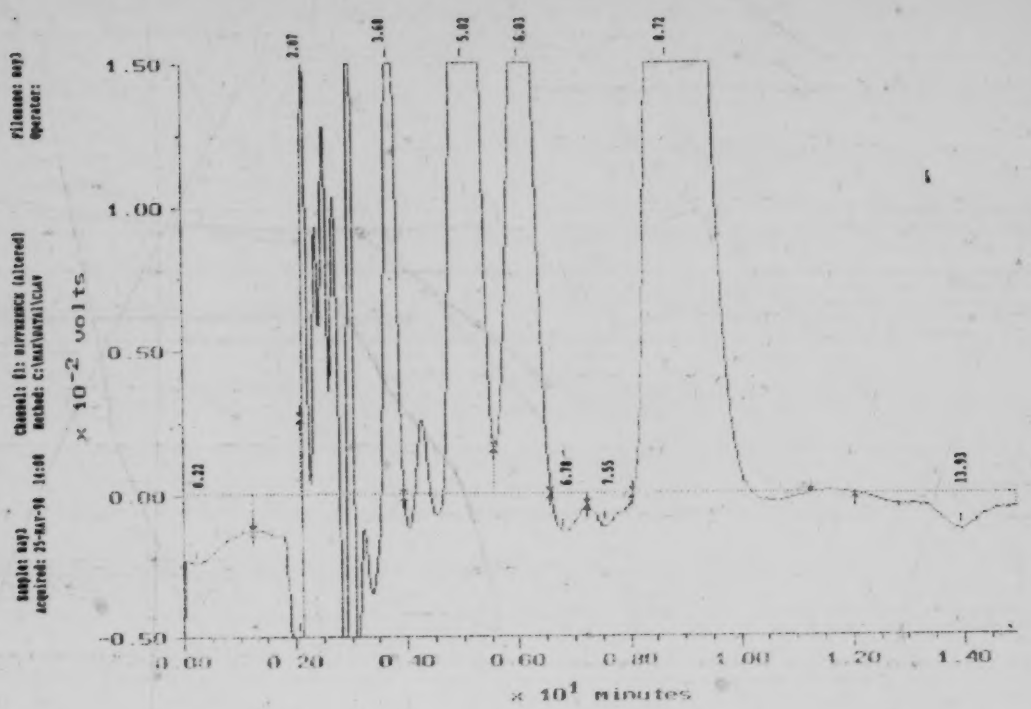
clavaminic acid (approx. 3.70 min. in Figure 15a), an intermediate in the pathway to clavulanic acid, is also not present when the *orf-2* mutant culture is grown on SA medium, which suggests that the *orf-2* disruption blocks the pathway to clavulanic acid prior to synthesis of clavaminic acid. No clavam metabolites were detected in either wild-type or *orf-2* cultures grown on SA medium, since SA medium does not provide the nutritional conditions neccessary for clavam production.

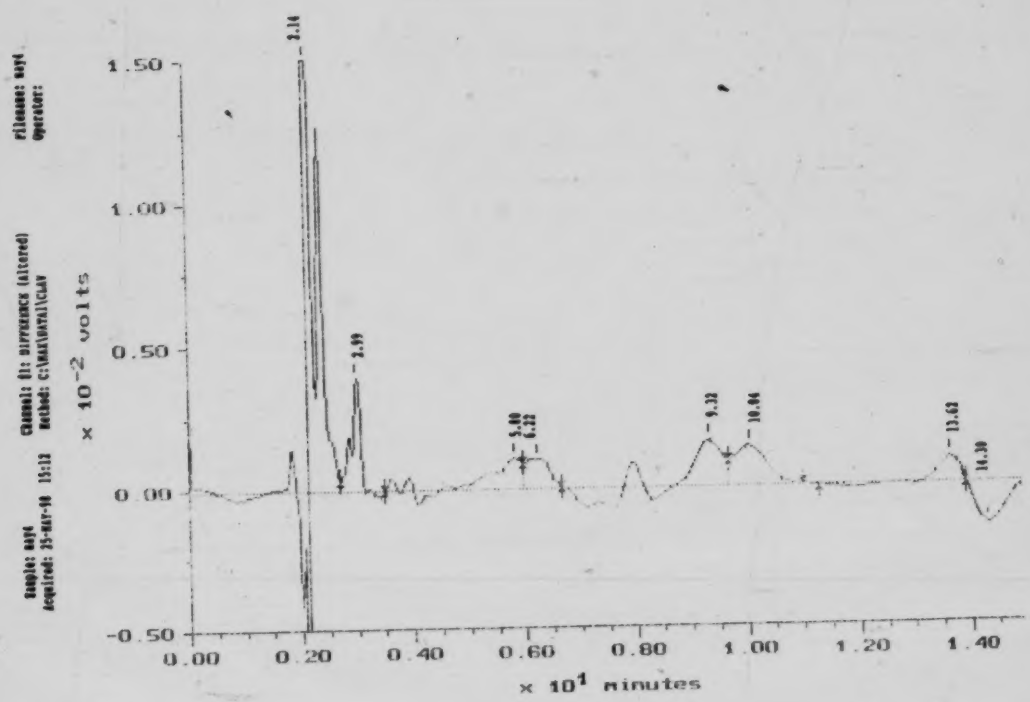
The *orf-2* mutant was also examined for clavulanic acid while grown under the nutritional conditions of soy medium. Samples were prepared in a the same manner as for SA cultures and examined for imidazole derivatized compounds. Figure 16a shows the HPLC chromatogram from wild-type *S. clavuligerus*, where a large peak corresponding to clavulanic acid (8.72 min.) is seen as expected and a peak corresponding to the intermediate clavaminic acid (3.68 min.). It can also be noted that there is a peak corresponding to the clavam metabolite clavam-2-carboxylate (3.68 min.) and the peak at 6.03 min. corresponds to 2-hydroxymethyl clavam (C. Anders, personal communication), although no authentic sample was available. Figure 16b shows the HPLC chromatogram of the *orf-2* mutant culture grown on soy medium. No clavulanic acid or clavam metabolites are detected in the *orf-2* mutant culture grown on soy medium.

III.3.5 Alanyl-clavam bioassay

HPLC analysis of *orf-2* mutant cultures could not detect the presence of clavulanic acid or any other clavam metabolites. It is possible to assay for the presence of the antimetabolite 2-alanyl-clavam in culture supernatant using an agar diffusion bioassay with *Bacillis sp.* ATCC 27860 as a sensitive indicator organism. Samples of culture supernatant were applied to filter disks on the surface of Davis minimal media inoculated with *Bacillis sp.* ATCC 27860. After overnight incubation, no zones of inhibition could be seen for the wild-type or *orf-2* mutant SA cultures. When samples from soy cultures were tested the *orf-2* mutant produced a zone of inhibition, corresponding to the presence of 2-alanyl-clavam, when grown on Soy medium although at reduced levels compared to wild-type

Figure 16: Clavulanic acid and clavam metabolite production by wild-type and *orf-2* mutants of *S. clavuligerus* on soy medium - HPLC analysis. A) Wild-type. B) *orf-2* mutant. 100 µl of culture supernatant was mixed with imidazole reagent (25 µl) and incubated at room temperature for 15 min to form imidazole-derivatized clavams. A 50 µL sample of the derivatized supernatant was then analyzed on a reverse-phase column (Bondapak-C18) with an isocratic buffer system consisting of 0.1 M KH_2PO_4-6% methanol, pH3.2 with detection at 312 nm.


1.50
1.00
0.50
0.00
-0.50
x 10-2 volts
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
x 10¹ minutes


1.50
1.00
0.50
0.00
-0.50
x 10-2 volts
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
x 10¹ minutes

(Table 5). Therefore, it appears that *orf-2* is required for wild-type levels of alanyl-clavam production, but *orf-2* is not essential for alanyl-clavam production when grown under the nutritional conditions of soy medium.

Table 5: 2-Alanyl-clavam production by wild-type and *orf-2* mutant of*Streptomyces clavuligerus.*

Growth[a] medium	Zone of Inhibition (mm)[b]	
	Wild-type	*orf-2* mutant
SA	0	0
Soy	39	16

a) Cultures were grown 72 hours in soy medium or SA medium before bioassay for 2-alanyl-clavam production.

b) 25 µl of culture supernatants were applied to filter paper disks on Davis minimal media (plus glucose) inoculated with *Bacillus sp.* ATCC 27860. Zones were measured after overnight incubation at 28°C.

IV DISCUSSION

The investigation of *orf-2* involved two major aspects: firstly, the specific mutation of *orf-2* via insertional inactivation in *S. clavuligerus*; and secondly, the characterization of the *orf-2* mutant to determine the effect of the inactivation of *orf-2*, specifically the effects on AHAS activity, cephamycin production and clavulanic acid production.

The pUC119 based plasmid pBB5.3-E contained *orf-2* as a 2.1-kb *Eco*RI-*Bgl*II fragment in the multiple cloning site which provided a simple and easy-to-use vector for the manipulation of *orf-2*. In order to disrupt the coding sequence of *orf-2*, the apramycin resistance gene was selected for insertion within the coding sequence of *orf-2*. It was chosen for three reasons: a non-homologous DNA sequence was needed to interrupt the coding sequence of *orf-2;* using an antibiotic resistance gene provides a useful marker; and most importantly the *apr* gene confers apramycin resistance in both *E. coli* and *Streptomyces*. The unique *Not*I restriction endonuclease site was chosen for the insertion of *apr* since it is centrally located within the 2.1-kb *Eco*RI-*Bgl*II *orf-2* fragment which allows sufficient upstream and downstream homologous *orf-2* sequences for efficient crossing over and recombination to occur with the wild-type copy of *orf-2*.

To accommodate the insertion of the *Nco*I flanked *apr* fragment into *orf-2* at the *Not*I site, a 14-bp oligonucleotide linker/adapter was made which contains a central *Nco*I restriction site as well as four base pair long 5′ overhangs which are complementary to the "sticky" ends of *Not*I digested DNA. Since only the overhanging ends are complementary to the *Not*I recognition sequence, ligation of the linker/adapter into the *Not*I site inserted a *Nco*I site, while altering the *Not*I site making it inactive. This provided an easy method for screening plasmids which accepted the linker/adapter into the *Not*I site. The resulting plasmid was then capable of being linearized by digestion with *Nco*I, while at the same time was no longer susceptible to digestion with *Not*I, since the recognition sequence was been altered.

Once the cloned copy of *orf-2* had been disrupted with *apr*, it needed to be transferred into *Streptomyces*. Since pKEO2Ap is a pUC-based vector not able to replicate within *Streptomyces*, O2Ap must be transferred to a *Streptomyces* vector. Two main possibilities were available: either link the entire plasmid pKEO2Ap to a *Streptomyces* vector making a plasmid able to shuttle between *E. coli* and *Streptomyces*; or remove the O2Ap fragment and insert it into a *Streptomyces* vector. Constructing a shuttle plasmid allows transformation of the ligation products into *E. coli* first, resulting in the quick and easy preparation of large amounts of purified plasmid for transformation into *S. lividans*. Transfering the insert to a *Streptomyces* vector kept the size of the plasmid construct small which may aid in transformation. Structural instability of *E. coli/Streptomyces* shuttle vectors was also encountered for some constructs. The latter method was chosen for the simple reason that it was the first attempted and it worked.

The *Streptomyces* vector pIJ486 was ultimately chosen to carry O2Ap to *S. clavuligerus*. It has a multiple cloning site containing *Eco*RI and *Hin*dIII recognition sites, which was suited for the insertion of O2Ap, since O2Ap could easily be removed from pKEO2Ap as a 3.5 *Eco*RI-*Hin*dIII fragment, with *Hin*dIII being just outside the right-hand end of the *orf-2* insert and in the remainder of the multiple cloning site of pUC119. The suitability of pIJ486 relies also on the fact that pIJ486 is segregationally unstable in *S. clavuligerus* when there is no antibiotic selection. This is important for the removal of the plasmid after homologous recombination and crossing-over with O2Ap carried on the plasmid and the genomic copy of *orf-2* has occurred. Once O2Ap has inserted into the genome it is stably maintained. The final goal was to have a strain with no intact *orf-2*. For this to happen, the wild-type copy of *orf-2* transferred from the genome to the plasmid must be removed. This was accomplished by the removal of the antibiotic from the growth medium that selects for the plasmid encoded resistance marker, allowing the segregationally unstable plasmid to be lost.

Direct transformation to *S. clavuligerus* with non-native DNA results in very low transformation frequencies due to restriction modification by *S. clavuligerus*. To circumvent this problem, plasmids are passed through *S. lividans* which has higher frequencies of transformation. The resulting plasmid DNA, since it comes from a related *Streptomyces* species, can then be transformed into *S. clavuligerus* with greater efficiency (Aidoo et al.,1994; Paradkar and Jensen, 1995).

The transformation of pIJO2Ap into *S. clavuligerus* was not the final step in creating an *orf-2* mutant. A rare event of homologous recombination and crossing over must occur between the plasmid encoded *orf-2*, which is disrupted by the *apr* gene, and the genomic copy of *orf-2*, followed by the loss of the plasmid. Primary transformants were selected for a single resistance marker, in this case the plasmid encoded a thiostrepton resistance marker. Experience has shown that the stress of both thiostrepton and apramycin, even in the presence of the respective resistance genes has been fatal to the regeneration of transformed protoplasts. Regenerated cells on the other hand are much healthier compared to protoplasts and could be screened for both selectable markers, (i.e. thiostrepton and apramycin resistance). It is assumed that homologous recombination and crossing over occurs during this round of growth and regeneration. The next round of growth and sporulation occurs without any antibiotic selection. Sporulation stimulated the loss of unselected plasmid, therefore the segregationally unstable pIJ486 based vector was lost, while the *apr* marker was stably maintained as genomic DNA residing within the now disrupted *orf-2*.

The *orf-2* mutants were selected by serially diluting harvested spores and plating onto sporulation medium (ISP-3) to allow growth of isolated colonies. The plates carrying the isolated colonies were then replica-plated onto ISP-3 with no antibiotic selection to ensure that efficient replica plating had been carried out, while replica-plating onto ISP-3 (5 µg/mL tsr) and ISP-3 (25 µg/mL apramycin) determined which of the isolated colonies on the master plates are true *orf-2* mutants. Colonies which were apramycin resistant and

thiostrepton sensitive were assumed to be true *orf-2* mutants for two reasons: firstly apramycin resistance could be maintained only if the *apr* gene inserted within the genomic DNA by the mechanism of homologous recombination of the *orf-2* sequences surrounding *apr* with that of the genomic copy of *orf-2*; and secondly, since the colonies were thiostrepton sensitive we can assume that the thiostrepton resistant plasmid resulting from the exchange of O2Ap and the genomic *orf-2* is lost. Colonies retaining free plasmid or colonies resulting from a single cross over would be both thiostrepton resistant and apramycin resistant. Approximately 8% of the colonies on the master plates arose from cells which had undergone homologous recombination, double crossing over, and loss of the plasmid resulting in apramycin resistant thiostrepton sensitive colonies.

To confirm that the *apr* disrupted copy of *orf-2* had replaced the wild-type *orf-2*, genomic DNA from the transformants was examined by Southern analysis and compared to that of wild-type. A complete replacement of *orf-2* with the disrupted *orf-2* from pIJO2Ap and the subsequent loss of the plasmid and intact *orf-2* was the desired event. Examination of DNA sequence surrounding *orf-2* and that of *apr* revealed restriction sites that would allow us to confirm that O2Ap had inserted in the place of *orf-2*. What was needed was a restriction site up and down stream of *orf-2* not found with the *apr* insert. *Eco*RI on the upstream side of *orf-2* and *Nru*I downstream of *orf-2* suited our needs. Digestion of total DNA with *Eco*RI and *Nru*I results in a 2.0 kb fragment encompassing *orf-2*, while the insertion of *apr* within *orf-2* results in an increase in the size of the *Eco*RI-*Nru*I fragment to 3.5 kb. Therefore Southern blot analysis of *Eco*RI and *Nru*I digested DNA from the mutants indicated that O2Ap had indeed inserted in the place of the genomic *orf-2*.

The above procedure excluded the presence of false mutants such as pIJO2Ap inserting as the result of a single crossing over event. This event would result in one of two combinations of two bands hybridizing to the *orf-2* probe. If the plasmid inserted into the genome as the result of crossing over event with the homologous sequences from the downstream portion of *orf-2*, the resulting bands hybridizing to a *orf-2* probe would be: a

3.5 kb fragment corresponding to O2Ap, and a 8.2 kb band comprised of *orf-2* and vector sequence. On the other hand if the plasmid inserted into genomic DNA as the result of a single crossing over event with homologous sequences from the upstream portion of *orf-2*, the resulting bands hybridizing to a *orf-2* probe would be: a 2.0 kb *Eco*RI-*Nru*I fragment corresponding to *orf-2*, and a 9.7 kb *Eco*RI fragment corresponding to pIJO2Ap. The latter combination of bands would also correspond to pIJO2Ap in the cell as free plasmid, although free plasmid should not be present within the cell following a round of growth and sporulation without the appropriate antibiotic selection. Probing an identical blot with an *apr* probe supported these conclusions since the *apr* probe hybridized to the same 3.5 kb fragment in the *Eco*RI-*Nru*I digested DNA from the *orf-2* mutants. We can therefore conclude that Southern analysis confirmed that the desired mutant had been isolated.

When this project was conceived the only information known about *orf-2* was that it was two ORFs downstream from the known end of the cephamycin gene cluster and three ORFs upstream from the only clavulanic acid gene identified (*cla*). Later it was shown that *orf-1* encodes a penicillin binding protein (pbp) which is partially responsible for the resistance of *S. clavuligerus* to penicillins and therefore *orf-1* (*pbpR*) is part of the cephamycin gene cluster putting *orf-2* immediately adjacent to the cephamycin cluster. Another lead as to the nature of *orf-2* was that it showed a high degree of similarity to the large subunit of AHAS enzymes from many species. Due to the tendency of genes for secondary metabolites to be clustered, it was assumed that *orf-2* would be involved in either cephamycin or clavulanic acid biosynthesis. With respect to the resemblance of *orf-2* to the large subunit of AHAS enzymes, it was hypothesized (S.E. Jensen, personal communication) that ORF-2 could be part of a secondary AHAS isozyme responsible for the flow of valine into the penicillin/cephamycin biosynthetic pathway.

The genes (*ilvB* and *N*) encoding the AHAS large and small subunit in related species are found adjacent to each other and only separated by a few base pairs. The ORF downstream of *orf-2* (*orf-3*) is also closely spaced with the separation being only 3 bp, but

orf-3 shows no resemblance to the small subunit of AHAS with respect to size or protein sequence. With *orf-3* showing no resemblance to the small subunit of AHAS, it seemed less likely that *orf-2* was an AHAS gene, since in all cases the large and small subunit are found together, even in enteric bacteria where more than one isozyme is found. If *orf-2* was indeed the large (catalytic) subunit of an AHAS enzyme, it would need to be active without the small (regulatory) subunit or be able to cannibalize the small subunit from the primary AHAS enzyme. Neither of the these two options seem likely, since all AHAS enzymes examined are composed of a large and small unit whose genes are adjacent to each other and possibly co-transcribed. If *orf-2* is part of a AHAS enzyme, it would seem to need a regulatory subunit to be active. If the new catalytic subunit produced by *orf-2* used the regulatory subunit from the primary AHAS, the new catalytic subunit might still be regulated in the same manner as the primary AHAS. Another option for this scenario, is that *orf-3* is indeed the regulatory subunit for *orf-2* in a AHAS enzyme even though it shows no similarity to known regulatory subunits. Recently, *orf-3* has been shown through gene disruption to be required for clavulanic acid biosynthesis (Paradkar, unpublished), thus the above theory seems unlikely.

AHAS enzymes from enteric bacteria have been investigated intensively and fully characterized, while few studies have been done on AHAS from *Streptomyces*. Since, at the time when the investigation into AHAS activity in *S. clavuligerus* was initiated, little was known of AHAS activity in *Streptomyces*, a protocol from Methods in Enzymology (De Felice et al., 1988) used for detecting two AHAS isozymes from enteric bacteria was adapted to provide a starting point for the examination of AHAS activity in *S. clavuligerus*. This method allowed a crude enzyme extract from *S. clavuligerus* to be examined for AHAS activity which correlated with that of isozyme I or isozyme III from *E. coli* K12. It seemed logical that if *S. clavuligerus* had more than one AHAS isozyme, they may correlate to the activity of isozyme I or III of *E. coli* K12, or at least show different activity profiles depending on pH or presence of FAD as had been observed for *E. coli* AHAS

isozymes. Figure 11 showed that the crude enzyme extract from *S. clavuligerus* did not have an AHAS activity that required additional FAD as a co-factor. The method of De Rossi et al. (1995) for extraction and assay of the AHAS enzyme from *S. avermitilis* expressed in *E. coli* contained FAD. However, whether FAD was a necessary co-factor for AHAS activity for this enzyme was not stated. In the assay of AHAS activity in *C. glutamicum* (Eggeling et al.,1987) FAD was not present in the enzyme assay, yet FAD was present in the extraction buffer. Sufficient levels of FAD for AHAS activity may have been present within the enzyme extract. Activity at pH 9.3 was approximately 35% of activity at pH 6.3 which also negates the possibility that AHAS of *S. clavuligerus* is similar to that of isozyme I of *E. coli*, since isozyme I is inactive at pH 9.3.

The supplementation of FAD at pH 6.6 and 9.3 did not effect AHAS activity. Therefore the addition of FAD to the reactions was omitted in further reactions. The pH optimum was then sought using two buffers to cover a full range of pH. The pH optimum was found to be pH 7.5 with both potassium phosphate and Tris-HCl buffers, with the higher activity in the phosphate buffer. Since there is only a single peak of enzyme activity, there is no evidence of multiple forms of the enzyme with different pH optima. This correlates well with *Corynebacterium glutamicum*, a close relative of *Streptomyces* spp., and *Streptomyces avermitilis,* both of which have only one AHAS enzyme. Further reactions were carried out using potassium phosphate buffer at pH 7.5. These conditions correlate with the findings of Eggeling et al.(1987), pH 7.8, and De Rossi et al. (1995), pH 7.0, used in their assays of AHAS activity from *C. glutamicum* and *S. avermitilis* respectively.

Although it was concluded that *orf-2* disruption did not alter AHAS activity, it was still conceivable that *orf-2* was involved in cephamycin biosynthesis due to its close proximity to the cephamycin gene cluster. Growth in SA (defined medium) limited the supply of valine to that produced by the branched chain amino acid pathway, therefore valine used for synthesis of ACV would need to be taken from the pool of primary

metabolites produced by *S. clavuligerus*. If *orf-2* is a secondary AHAS used to overcome valine limitation, the disruption of its activity should decrease the availability of valine for the synthesis of penicillin and cephamycin. Agar diffusion bioassays of culture supernatant using *E. coli* ESS as a sensitive indicator to β-lactam antibiotics showed that disruption of *orf-2* did not alter antibiotic production in SA or soy medium. Since AHAS activity and β-lactam antibiotic synthesis were not altered by disruption of *orf-2*, we can conclude that *orf-2* is not involved in cephamycin biosynthesis. Open reading frames downstream from *orf-2* (orf-4 and 5) which have been disrupted (Aidoo et al, 1993; Paradkar and Jensen, 1995) also showed unaltered cephamycin biosynthesis.

Characterization of clavulanic acid biosynthesis by the *orf-2* mutant involved analyzing the supernatant of either SA or soy medium cultures. In previous work on clavulanic acid genes, it was found that disruptional mutation of *cla* or *cs2* resulted in a greatly reduced levels or no clavulanic acid when grown under the nutritional conditions of SA medium, while clavulanic acid was still produced, although at reduced levels when grown on soy medium (Aidoo et al, 1994; Paradkar and Jensen, 1995). The leaky phenotype of the *cs2* mutant when grown on soy medium is explained by occurence of a second gene encoding clavaminate synthase (*cs1*) which is under different regulatory control than that of *cs2*. CS1 is produced when *S. clavuligerus* is grown in soy medium, while expression of *cs1* could not be detected when grown in SA medium (Paradkar and Jensen, 1995). This explains why, when *cs2* is inactivated, clavulanic acid is still produced in soy medium. Unpublished data also indicate that there is a second copy of *cla*. Since the *orf-2* mutant did not show a similar phenotype to that of *cla* and *cs2* mutants, it could not be concluded that there is a second gene with an analogous function to that of *orf-2*, as is the case with *cla* and *cs2*. It has been hypothesized that genes encoding enzymes specific for clavams other than clavulanic acid are present elsewhere on the chromosome and the possible reason for the duplication of genes is that the pathway to all clavams is parallel to the point of clavaminic acid.

Many pieces of evidence point to *orf-2* being responsible for one of the early steps in the pathway to clavulanic acid: *orf-2* appears to be under the same nutritional control as *cla* and *cs2*; as well, Paradkar and Jensen (1995) assumed that *orf-2* is co-transcribed on a 5.2 kb transcript along with *orf-3*, *cla*, and *cs2*. The products of *cla* and *cs2*, PAH and CS2 respectively, are enzymes responsible for converting the earliest known β-lactam ring-containing intermediate (deoxyguanidinoproclavaminic acid) of the clavulanic acid pathway to clavaminic acid. Therefore since *orf-2* is under the same control as genes responsible for the early steps in clavulanic acid biosynthesis up to clavaminate, it is possible that *orf-2* encodes an enzyme responsible for one of the early steps to clavaminate.

The resemblance of *orf-2* to the large (catalytic) subunit of AHAS enzymes may also shed some light on the function of ORF-2. AHAS catalyzes two parallel reactions: the irreversible decarboxylation of pyruvate and its condensation with a second molecule of pyruvate to form acetolactate in the pathway to valine and leucine; and the irreversible decarboxylation of pyruvate and its condensation with α-ketobutyrate to form acetohydroxybutyrate. In broader terms, AHAS catalyzes the condensation of two small molecular weight compounds, which is very similar to what must occur in the first step (or steps) in the clavulanic acid pathway in which pyruvate and arginine are somehow combined to form the first identifiable intermediate in the clavam pathway, N2-(2-carboxyethyl)arginine.

The disruption of *orf-2* with *apr* caused interruption of clavulanic acid biosynthesis while grown on SA medium. Some concern exists whether the possibility that *orf-2* is not essential at all for clavulanic acid biosynthesis. The insertion of *apr* within *orf-2* may have caused a polar effect on *orf-3*, since there are only 3 bp separating *orf-2* and *orf-3*. *Orf-2* and *orf-3* may be co-transcribed and the insertion of *apr* within *orf-2* may have disrupted the transcription of *orf-3* as well. Therefore the *orf-2* mutant may not have active gene products from either *orf-2* and *orf-3*. Attempts were made to determine if *orf-3* was being

transcribed in the *orf-2* mutant using Northern blot analysis, but the investigation was inconclusive.

Some evidence exists which suggests that the interruption of clavulanic acid biosynthesis in the *orf-2* mutant is not just the result of a polar mutation on *orf-3*. When *apr* was inserted in the reverse orientation to disrupt the *lat* gene, there was still approximately 25% expression of the downstream gene as the result of reading through from the upstream promoter (Dylan Alexander, unpublished results). Futhermore, the specific disruption of *orf-3* caused a less severe reduction of clavulanic acid biosynthesis than that of the disruption of *orf-2*, therefore at least some effect must be due to the disruption of *orf-2*, although it can not be ruled out that there is some kind of additive effect.

Future studies would include S1 transcriptional analysis to determine whether *orf-3* is still transcribed in the *orf-2* disruptant. Does the insertion of *apr* within *orf-2* cause polar effects on the transcription of *orf-3*? If the *apr* gene is interfering with transcription *of orf-3*, the interference may be alleviated by the construction of a new *orf-2* disruptant with *apr* in the opposite orientation. Along the same lines, a new *orf-2* disruptant with the *apr* gene removed could be made which would preserve transcription yet still produce a non-functional *orf-2* protein. The production of alanylclavam while no other clavams are detected was also of interest. The bioassay procedure was difficult in the sense it did not provide easily reproducible results. A definitive answer on whether alanylclavam is present in the orf-2 mutant extract may be able to be determined by imidazole derivatization and HPLC analysis, although the method for clavulanic acid does not work for alanylclavam. Possibly an alternate buffering system or column way be available to separate alanylclavam from background noise. It would also be of interest to investigate whether *orf-2* has a homologue as is the case with *cs2* and *cla*. It has not yet been determined if *cs1* and the homologue for *cla* are linked and form a cluster of clavam genes. If a homologue for *orf-2* is indeed present, it would most likely be associated with other clavam genes.

The next and final phase of investigation into *orf-2* would be to ascertain the biochemical function of the *orf-2* gene product. This may be achieved via over-expression of the *orf-2* gene and purification of the protein, then using a range of radioactively labeled substrates determine the biochemical activity of ORF-2.

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